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THE *IN VITRO* INTERACTIONS BETWEEN
TUBULIN AND HIV-1 REV REQUIRE
REV'S MULTIMERIZATION AND ARGININE-
RICH MOTIFS

A dissertation submitted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

By

AMIT SHARMA
M.Sc., Bangalore University, India, 2001

2009
Wright State University

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November 12, 2009

I HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER MY SUPERVISION BY Amit Sharma ENTITLED The *in vitro* Interactions between Tubulin and HIV-1 Rev require Rev's Multimerization and Arginine Rich Motifs BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Doctor of Philosophy.

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ABSTRACT

Sharma, Amit. Ph.D., Biomedical Sciences Program, Wright State University, 2009.
The *in vitro* Interactions between Tubulin and HIV-1 Rev require Rev's Multimerization and Arginine Rich Motifs.

Rev is a 13 kDa regulatory HIV protein essential for viral replication. It *trans*-activates expression of late viral proteins by multimerizing onto target mRNA and promoting their export into the cytoplasm. During attempts to find the solution conditions needed to study Rev structure, Watts *et al.* (2000) discovered that Rev depolymerizes microtubules (MTs) *in vitro* through the formation of ringed tubulin intermediates called Rev-tubulin toroids (RTTs). Rev interactions with MTs are specific and are thought to mimic the mechanism of Kinesin-13 proteins, themselves potent MT depolymerases that regulate the assembly of the mitotic spindle. If Rev and Kinesin-13 proteins share a common mechanism, then Rev mediated MT depolymerization and RTT formation will require Rev multimerization and its arginine-rich motif (ARM).

If Rev multimerization is essential, then multimerization defective mutants should not depolymerize MTs and form RTTs. To test this hypothesis, a combination of sedimentation, gel filtration and visual assays were used to compare the activities of wild-type Rev with the multimerization defective mutant Rev M4 (M4). Both wild-type Rev and M4 are able to bind tubulin heterodimers and form high molecular complexes. However, these complexes are not RTTs. M4 also binds GMPCPP-stabilized MTs but unlike wild-type Rev, it neither depolymerizes MTs nor forms RTTs. These data show that Rev multimerization is important for MT depolymerization although it is unclear whether it is involved in targeting Rev to MT ends or provides the force required for

depolymerization. Because M4 promotes MT bundling, this mutation is concluded to subtly affect Rev tertiary structure such that the relative orientation of Rev monomers within a multimer is altered in a manner that allows MT cross-linking.

These same assays were also used to test the hypothesis that the ARM is also important for Rev-tubulin interactions. In this instance, the activities of wild-type Rev were compared to the M6 mutant (M6), a well-characterized substitution-deletion mutant in the ARM predicted to perturb binding, depolymerization and RTT formation. The M6 mutation affects RTT formation when mixed with tubulin heterodimers, reducing both ring size and the amount of complexes that can be sedimented. In addition, M6 is unable to depolymerize GMPCPP-stabilized MTs through an apparent inability to bind MT. Results presented here suggest Rev possesses only a single MT binding motif present in the ARM. Some residues in this region are critical for binding MT ends where depolymerization occurs. These conclusions are also consistent with the hypothesis that Rev interacts with MTs by a mechanism shared with Kinesin-13 proteins, themselves potent cellular MT depolymerases.

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ABBREVIATIONS

1. A	Adenine
2. AIDS	Acquired Immune Deficiency Syndrome
3. Arg/R	Arginine
4. Asn/N	Asparagine
5. Asp/D	Aspartic Acid
6. ARM	Arginine Rich Motif
7. ATP	Adenosine triphosphate
8. C	Cytosine
9. CD	Circular Dichroism
10. CRM-1	Chromosome Region Maintenance-1 (Exportin- -molecule)
11. C-terminal	Carboxy terminal
12. DMS	Di-Methyl Sulfate
13. DTT	Dithiothreitol
14. EMSA	Electrophoretic Mobility Shift Assay
15. FRET	Fluorescence Resonance Energy Transfer
16. G	Guanine
17. GDP	Guanosine Diphosphate
18. Gln/Q	Glutamine
19. Glu/E	Asparagine
20. GTP	Guanosine triphosphate
21. GMPCPP	Guanosine -5'-[(α,β)-methylene]triphosphate (Slowly-

-hydrolysable analog of GTP).

22. HIV-1	Human Immuno Deficiency Virus-1
23. HLH	Helix-Loop-Helix
24. IF	Immuno-Florescence
25. Ile/I	Isoleucine
26. kDa	Kilo-Dalton
27. Leu/L	Leucine
28. Lys/K	Lysine
29. M4	Rev M4
30. M6	Rev M6
31. MDa	Mega Dalton
32. MCAK	Mitotic Centromere-Associated Kinesin
33. Mg ⁺⁺	Magnesium
34. MT(s)	Microtubule(s)
35. NMR	Nuclear Magnetic Resonance
36. Nup	Nucleoporin
37. <i>N</i> -terminal	Amino terminal
38. PMSF	Phenyl Methyl Sulphonyl Fluoride
39. Rev	Regulator of Envelope Region/Regulator of Virion- -particles/wild-type Rev
40. RNA	Ribo-Nucleic Acid
41. RRE	Rev-Response Element
42. RTT	Rev-Tubulin Toroids

43. S200	Superdex-200
44. SLIIB	Stem-Loop II-B
45. SPR	Surface Plasmon Resonance
46. TEM	Transmission Electron Microscope
47. U	Uracil
48. WCE	Whole Cell Extract

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DEDICATION

This thesis is dedicated to my parents, brother and wife who opened the doors of wonderful possibilities for me through their constant love, support and encouragement.

This thesis is also dedicated to Dr. Miller, who once told me that there are two kinds of people in this world- “ One who are genius and others who are hard working”.

INTRODUCTION

Human Immunodeficiency Virus-1 (HIV-1) is a causative agent of Acquired Immune Deficiency Syndrome or AIDS (Malim and Pollard, 1998; Hope *et al.*, 2001). Millions of people die of AIDS every year. Although the current therapeutic strategy slows the intensity of AIDS infection there is still no known cure for AIDS. Consequently, research aimed at understanding the regulation of infection and identifying novel HIV targets is important. Rev is such a target.

Rev is a 116 amino acid, arginine-rich protein essential for replication of the HIV (Pollard and Malim, 1998). It activates late viral gene expression by promoting nuclear export of under-spliced viral mRNAs normally retained and degraded in the nucleus (Figure 1) (Malim and Cullen, 1989a; Malim *et al.*, 1991). Rev has an arginine-rich motif (ARM, residues 34-62) that recognizes these RNAs by binding to a 351-nucleotide, stem loop structure present within an intron called the Rev Response Element (RRE) (Figure 2). Mutations in the ARM inhibits expression of late viral genes by inhibiting RRE binding as measured by functional assays monitoring expression of late viral gene products such as gag and env and assays monitoring RRE binding, e.g., gel retardation assays and surface plasmon resonance (Malim *et al.*, 1991, Pollard and Malim, 1998; Van Ryk and Venkatesan, 1999). Amino acid substitutions and/or deletions of clustered arginine residues in the ARM, amino acids 38-39 and 41-44 (e.g., M5 and M6 mutations, respectively), profoundly limit Rev binding to RRE (Malim *et al.*, 1989b; Hammerschmidt *et al.*, 1994; Pollard and Malim, 1998; Brice *et al.*, 1999) (Table 1). Additional RNA footprinting experiments confirm arginines 38, 39, 50, and 66 are important for binding the RRE (Jensen *et al.*, 1995).

Figure 1: The Biological Function of Rev. **A)** HIV-1 Rev transcribes into completely spliced and RRE containing incompletely spliced transcripts. **B)** Incompletely spliced transcripts are not transported into the cytoplasm and are degraded. **C)** Completely spliced transcripts are transported to the cytoplasm and are translated into Rev, Tat and Nef. **D)** Translated Rev is transported to the nucleus by binding Importin β . **E, F)** Rev binds and multimerizes on to RRE. **G)** Multimerization stimulates Crm-1 binding to Rev's NES. This complex is stabilized by Ran-GTP binding and enables export of Rev-RRE complexes. RRE containing mRNAs are then translated into the structural proteins (Gag, Env, Pol, Vif, Vpr) of the virus.

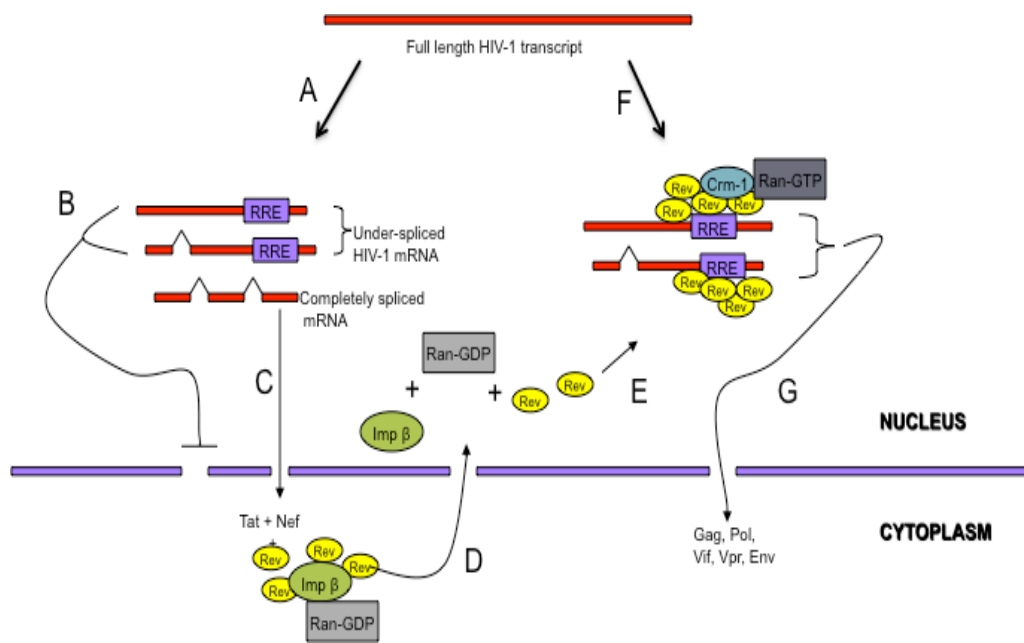
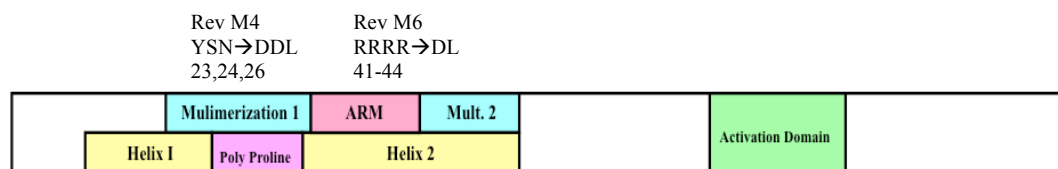


Figure 2: Domain Structure of Rev. **A)** The domain structure of Rev showing the multimerization motif-1 residing in helix-1, a poly-proline loop, and the ARM and multimerization motif-2 (mult.2) present in helix-2. This figure also shows the locations of the NES and the M4 and M6 mutations. **B)** Shows the sequence similarity shared between Rev and the motor domain of MCAK. Residues marked in red are identical and those marked in blue are conserved substitutions.

A.



B.

HIV-1 Rev: 34 TRQARNRNRWRERQRQIHSISERILSTYLGRSAEP
MCAK:506 TASADRITRMEGAEINRSLLALKECIRALGQNKSETP

Table 1: Summary of mutants used in this study. The table shows the mutants of Rev and their functional properties (Malim and Cullen, 1991).

Rev Mutant	Mutation	Multimerization	RRE Binding
Rev M4	YSN _(23,25,26) → DDL	-	+
Rev M5	RR _(38,39) → DL	+	-
Rev M6	RRRR ₍₄₁₋₄₄₎ → DL	+	-
Rev M7	SIS ₍₅₄₋₅₆₎ → I	-	+

The RRE consist of a series of intramolecular double stranded RNA stems and single stranded loops found only within the 3' introns of viral RNAs (Daly *et al.*, 1989; Malim *et al.*, 1989; Mann *et al.*, 1994; Malim and Pollard, 1998). Rev binds one such stem-loop structure called SLIIB with ≈ 1 nM affinity (Daly *et al.*, 1989; Malim *et al.*, 1989; Mann *et al.*, 1994; Malim and Pollard, 1998). Specifically, it binds the stem of SLIIB that forms a distorted helix due to non-Watson-Crick purine-purine base pairing (G47-A73 and G4-G71) (Bartel *et al.*, 1991; Malim and Polard, 1998). Initial binding of a Rev monomer is mediated by nonspecific electrostatic interactions between arginine residues of the ARM and negatively charged phosphate backbone of RRE as determined by gel retardation and NMR (Daly *et al.*, 1993; Battiste *et al.*, 1996; Belasco *et al.*, 1996; Van Ryk and Venkatesan, 1999).

Rev binding is insufficient to stimulate export of RRE-containing transcripts; however, binding the SLIIB site stimulates partially cooperative binding of four additional Rev monomers (Malim *et al.*, 1989; Daly *et al.*, 1993; Battiste *et al.*, 1996; Belasco *et al.*, 1996; Van Ryk and Venkatesan, 1999). Multimerization then stimulates binding of the nuclear export factor Crm1 (also called Exportin) that binds a nuclear export sequence (NES) present towards the Rev C-terminus (Fornerod *et al.*, 1997; Fukuda *et al.*, 1997) (Figure 1). This transport complex, stabilized by the binding of the Ras-like nuclear GTPase Ran bound to GTP (Fukuda *et al.*, 1997), is exported into the cytoplasm via nuclear pore complexes through interactions with Nup214 and Nup98 (Fornerod *et al.*, 1997; Zolotukhin and Felber 1999).

Rev Structure

To better understand the mechanism of Rev structure and facilitate development of antiviral therapies that target Rev function, many labs have attempted to determine Rev's tertiary structure. The structure is not completely known largely

due to Rev's tendency to aggregate and precipitate precluding structural studies (Heaphy *et al.*, 1991; Wingfield *et al.*, 1991; Watts *et al.*, 1998). Purified Rev protein polymerizes into filaments 8 nm in diameter when incubated with RRE at 40-160 $\mu\text{g/mL}$ (Wingfield *et al.*, 1991). At 0°C in the absence of RRE, Rev polymerizes into hollow filaments 20 nm in diameter with a 5-7 nm diameter axial channel, as viewed by standard TEM (Heaphy *et al.*, 1991; Wingfield *et al.*, 1991). Higher resolution STEM analyses show Rev filaments are a six-start helix with 31 dimers in two turns (Watts *et al.*, 1998). Within the filaments, the Rev dimer has a diameter of 3.2 nm and a height of 2.2 nm. The outer and inner filament diameters were approximately 14.8 nm and 10.4 nm. The outside of the filament appears smooth whereas the inner surface has extrusions resembling a 'top hat.' (Watts *et al.*, 1998).

Computer modeling and structural studies that include circular dichroism (CD) and solid-state NMR studies reveal the *N*-terminal half of unliganded Rev assumes a helix-loop-helix structure (amino acids 9-24, 25-33, and 34-62, respectively) with ARM residing in the second helix (residues 34-62) (Auer *et al.*, 1994; Blanco *et al.*, 2001) (Figure 2). CD analyses of two *C*-terminal deletion mutants of Rev, Rev Δ 68-112 and Rev Δ 92-112, show that Rev contains 46-49 residues in α -helical conformation in the *N*-terminus (Auer *et al.*, 1994). Deletion of amino acids 8-26 from Rev causes significant reduction in helicity and also inhibits Rev's ability to distinguish between RRE and non-specific RNA by 50-fold (Auer *et al.*, 1994). Conversely residues 75-93 are 40% β -sheet and less than 20% α -helical in nature. Using Raman spectroscopy, the *N*-terminus of Rev was found to be α -helical with the helices oriented in alignment with the longitudinal filament axis (Watts *et al.*, 1998). Using solid state NMR, Blanco *et al.* (2001) found backbone dihedral angles between

residues Leu13 and Val16 and residues Arg39, 42, 43, and 44 are consistent with the presence of α -helices.

Havlin *et al.* (2007) using transmission electron microscopy, atomic force microscopy and solid state NMR examined the secondary structure of Rev when it is bound to 45 bp of SLIIB. Intriguingly, although the morphology of Rev filaments change when it is bound to the RRE (Wingfield *et al.*, 1991; Havlin *et al.*, 2007), the tertiary structure of Rev does not change detectibly (Havlin *et al.*, 2007).

Structural basis of Rev-RRE interactions

The first glimpse of Rev-RRE structure came when Battiste *et al.* (1996) used solution NMR to examine the complex formed between residues 34-50 of Rev and SLIIB. Their analyses confirm these amino acids assume a helical conformation. They showed that Asn40 and Arg44 interact with the nucleotides U45, G46, G47, and A73 in the major groove of the RRE. Arginines at positions 38, 41-43, 46 and 48 also make hydrogen bonds and/or electrostatic interactions with the phosphate backbone of the RRE. Thr34 that forms hydrogen bonds with G47 further stabilizes these interactions. Arginines 46, 48 and 50 appear to make van der Waal and electrostatic contacts that orient the C-terminal helix in the major groove of the RRE (Battiste *et al.*, 1996).

Various groups have conducted mutational analyses and studied the role of ARM in Rev-RRE interactions. For example, Belasco *et al.* (1996) created an expression vector where lacZ coding sequences were fused to an RRE such that expression of β -galactosidase was dependent upon Rev function through RRE. By placing point mutations in the RRE-LacZ or Rev expression constructs, they were able to identify nucleotides and amino acids important for Rev function, respectively. They proposed Rev makes multiple contacts with the RRE. Gln36, Gln51, Asn40,

Glu47 of ARM are buried in the major groove of the SLIIB. Asn40 contacts the SLIIB, and Glu47 and Gln51 of ARM lie close to the phosphodiester backbone of the SLIIB. The series of arginines (34, 35, 38, 39, and 44) were postulated to be accessible for direct interaction with the RNA.

Mutating individual arginines in the ARM does not affect Rev's ability to bind RRE or localize in the nucleus suggesting that deletion of four arginines in the M6 may create a change in the conformation of ARM that prevents it from binding the RRE (Hammerschmid *et al.*, 1994) (Table 1). Alternatively, the deletion of these four arginines as a whole may create a critical loss of electrostatic interactions with phosphate backbone of RRE as these residues form a part of residues that establish electrostatic contact with the RRE backbone as proposed by Battiste *et al.* (1996).

The ARM also contains the nuclear localization sequences (NLS) (Malim and Pollard, 1998; Hope, T.J., 1999). The NLS interacts with importin β that targets Rev to the nucleus (Truant and Cullen, 1999). The presence of NLS along with the C-terminal NES confers Rev the ability to shuttle in and out of nucleus. Because the RRE and importin β binding sites are overlapping, Rev bound to RRE can only be exported. Rev cannot be imported into the nucleus until the RRE dissociates thus preventing the re-import of RRE mRNA back in the nucleus. Whereas most ARM mutations affect both import and RRE binding, mutating Trp45 affects import and not RRE binding (Hammerschmid *et al.*, 1994).

Rev multimerization

Many labs have found a correlation between the extent of multimerization on the RRE as detected by EMSA and the extent that RRE-containing transcripts are translated (Malim *et al.*, 1989, Cook *et al.*, 1991; Daly *et al.*, 1993). Earliest evidence of Rev multimerization were obtained by Malim *et al.* (1989) who identified

mutations that inhibited Rev function without inhibiting RRE binding. One of these harbored mutation in the NES (Rev M10). EMSA's revealed the other two mutants, M4 and M7, were unable to form multimeric structures on the RRE. They co-transfected Tat expression vector along with wild-type Rev, M4, M7 and M10 in COS cells. They observed Tat expressed as a truncated 72 amino acid protein in the presence of wild-type Rev, showing that wild-type Rev was exporting partially spliced Tat mRNA to the cytoplasm. They however observed expression of a full-length 86 amino acid Tat protein in the presence of M4, M7 and M10 showing they are unable to export unspliced RNAs. Subsequent proviral rescue experiments found that wild-type Rev and not M4, M7 and M10 was able to rescue Rev deficient HIV replication in COS cells, further suggesting the importance of mutated residues in Rev's function (Malim *et al.*, 1989).

Subsequent mutational analyses refined the requirement of two multimerization motifs to residues 12-34 (multimerization motif-1) and residues 51-63 (multimerization motif-2, Figure 2) (Malim *et al.*, 1989, Auer *et al.*, 1994; Thomas *et al.*, 1997). Both are rich in hydrophobic residues and are a part of the *N*-terminal helix-loop-helix domain (Figure 2) (Auer *et al.*, 1994, Thomas *et al.*, 1998, Blanco *et al.*, 2001). Thomas *et al.* (1998) transfected a Rev-dependent expression plasmid into cells expressing Rev to measure the ability of different multimerization mutants to trans-activate expression of a reporter gene. This reporter plasmid encodes gene for chloramphenicol acetyltransferase (CAT) fused to the RRE and is positioned within an intron. In the presence of Rev activity, the CAT-RRE transcript will be exported before the CAT coding sequences are spliced out. Using this strategy they found that amino acids Ala15, Val16, Leu18, Ile19, and Leu22 in multimerization motif-I and residues Ile52, Ile59, and Leu60 in multimerization motif-II are critical for Rev

function. They predicted that the two helices bind each other and residues 12-23 in helix-1 and Ile52, 55 and 59 in helix-2 form a hydrophobic patch creating a site able to bind a second Rev monomer (Thomas *et al.*, 1997). This model is supported by Jensen *et al.* (1997). They digested Rev with different proteases and used radiolabeled monoclonal antibodies with defined binding sites to map antigenic sites. By comparing protein footprints, they showed that Rev *N*- and *C*-termini must lie close to each other.

Although many mutants that inhibit multimerization on the RRE have been identified, two mutations have been extensively studied: M4 and M7 (Table 1; Malim *et al.*, 1989, Daly *et al.*, 1995; Thomas *et al.*, 1998; Brice *et al.*, 1999). Malim *et al.* (1991) compared the ability of multimerization mutants M4 and M7 with wild-type Rev to bind radiolabelled RRE. The results of EMSA's demonstrated that M4 and M7 were unable to form the characteristic high molecular weight complexes normally formed by wild-type Rev suggesting that M4 and M7 are deficient in multimerization. Brice *et al.* (1999) attributed the deficiency of Rev M7 in multimerization to significant structural disruptions introduced due to double deletions. M4 was found to be defective in forming higher order multimers due to localized structural changes (Brice *et al.*, 1999). Daly *et al.* (1993) demonstrated that Rev-RRE complex consists of at least 2-8 Rev molecules at equilibrium. Moreover, complexes of two or more Rev monomers dissociate from RRE less readily than a single bound monomer suggesting that binding of two or more Rev molecules stabilizes Rev-RRE complexes. These results are confirmed by findings of Charpentier *et al.* (1997) who used dimethyl sulfate (DMS) to study Rev binding sites. DMS methylates the nitrogen bases of RNA molecules. In turn, methylation can be detected by primer extension during PCR. Methylation is prevented by protein bound to RNA and thus

can be used to detect the region in RRE where Rev binds. Peterson and Feigon (1996) have demonstrated that binding of 28 amino acids of ARM causes the change in conformation of backbone of the high-affinity binding site in RRE. This was confirmed by the findings of Charpentier *et al.* (1997) who found that binding by two Rev monomers induces a conformation change in RRE that facilitates Rev oligomerization *in vivo*.

Belasco *et al.* (2001) used a different strategy to investigate multimerization. Two Rev binding sites were introduced upstream of the ribosome binding site (RBS) of lacZ reporter containing the single copy of wild-type RRE SLIIB. The second of the two Rev binding sites, adjacent to the RBS was mutated in a way that it could not bind Rev. The logic of this design is that if Rev binds the Rev binding site upstream and multimerizes, it will interfere with the binding of ribosome to the reporter mRNA and prevent its translation. Using this strategy, they found residues Val16, Leu18, Ile19, and Leu22 and residues Arg48, Gln50, Ile52, Ile55, Ile59, and Leu60 are important for Rev multimerization on RRE.

Van Ryk and Venkatesan (1999) used SPR to measure Rev-RRE interactions. RRE was adhered to the sensor chip and Rev flowed over the chip. Through the use of different length of RRE and different titrations of Rev, their results demonstrated that Rev-RRE binding was partially cooperative and that multimerization was not a prerequisite for binding. In contrast to the findings of Daly *et al.* (1993), Van Ryk and Venkatesan (1999) also demonstrated that the C-terminus was not required for multimerization. Deletion of residues 87-116 did not affect the binding to the truncated form of RRE (containing the high affinity SLIIB) and bound with same affinity as wild-type Rev. Further, Daugherty *et al.* (2008) have characterized an additional site stem IA on RRE where oligomerization is inhibited by mutations

R41A, R42A and W45A. They summarized that Rev uses a combination of binding modes (binding to Stem IIB, Stem IA) to bind RRE.

In an effort to show the importance of multimerization of Rev *in vivo*, Madore *et al.* (1994) developed a reporter assay similar to two-hybrid system where CAT expression is dependent upon Rev oligomerization. The reporter assay consisted of a CAT indicator gene fused to high affinity Rev binding site-SLIIB. Their results demonstrated that Rev multimerization occurs *in vivo*. Both the M4 and M7 mutations inhibited CAT expression although to different extents. M4 stimulated CAT expression that was only 40% of wild-type Rev levels, M7 retained only 10% ability. Both mutants were concluded to be multimerization deficient.

Jain and Bellasco (2001) were able to differentiate between three classes of mutants based on their varying ability to multimerize on RRE in gel retardation assays. Class one mutants bind the RRE as monomers but are defective in forming dimers and higher order multimers. For example, mutating amino acids 18 or 55 did not affect the K_d of the Rev monomer to bind the RRE but reduced the affinity for dimer binding 10-fold. Class two mutants readily form dimers but show reduced higher order multimerization. Class three mutants show defects in all stages of RRE binding which were concluded to be structurally defective. Trikha *et al.* (2005) used a reporter construct (HIV envelope derived reporter under CMV early promoter) and demonstrated that trimerization defective mutants (class two mutants) (L12E, V16D, L60R; Belasco *et al.*, 2001) were deficient in nuclear export of reporter construct showing multimerization is essential for nuclear export *in vivo*.

Daelmans *et al.*, (2004) used FRET and FRAP to show Rev multimerization occurs in both the cytoplasm and nucleus. They observed FRET interactions between cytoplasmic mutants fused to GFP and BFP and observed FRET in cytoplasm. They

also observed localization of Rev M5-GFP in the nucleus when expressed with wild-type Rev-BFP construct confirming Rev multimerization *in vivo* and that ability to multimerize does not rely on presence of ARM.

Rev-tubulin interactions

Rev has the propensity to form long filaments *in vitro* that despite retaining the ability to bind RNA, have the tendency to aggregate (Heaphy *et al.*, 1991; Wingfield *et al.*, 1991). These filaments can be depolymerized by polyanions such as poly-G, poly-dG and poly-glutamate suggesting that Rev may be solubilized in an negatively charged environment. In an attempt to find the solution conditions that allow determination of Rev's structure, Watts *et al.* (2000) reasoned tubulin an ideal candidate for structural studies. Tubulin is acidic 50 kDa protein with a C-terminal poly-glutamate tail (also known as E-hooks) exposed on the surface. To determine the solution conditions needed to study Rev structure, they found that Rev depolymerized MTs into bilayered ring like structures called Rev-tubulin toroids (RTTs). The RTTs are also formed with colchicine treated tubulin heterodimers. RTTs are 3-4 MDa in molecular weight and have a mean outer diameter of 47 nm. STEM analyses suggested that tubulin lines the outside of RTT with Rev lining the inside. The apparent stoichiometry of Rev and tubulin is 1:1.

The interaction between Rev and tubulin is specific as RTTs form when Rev is mixed with rat or bovine tubulin. RTTs do not form when tubulin is incubated with other basic proteins like histones, lysozyme and poly-lysine. RTTs are stable and unaffected by changes in pH (6-8) and temperature (4 °C and 25 °C). RTT formation is unaffected at salt concentration as high as 200 mM. The formation of RTTs requires Mg⁺⁺ ions. In the presence of chelating agents, Rev still depolymerizes MTs but RTTs do not form. Instead, a soluble ≈110 kDa complex corresponding to one

Rev monomer and one tubulin heterodimer forms. RTTs also form with tubulin treated with subtilisin, protease that cleaves the C-terminal polyglutamate tail of tubulin, showing that Rev-MT interactions are not limited to simple electrostatic interactions. RTT formation occurred with both taxol stabilized MTs and colchicine treated heterodimers suggesting Rev's ability to recognize specific surface exposed residues of tubulin. It also suggests that the binding site for Rev is different from taxol and colchicine binding sites. RTT formation is however blocked when tubulin dimers are preincubated with the antimitotic drug maytansine. Since maytansine is known to bind β -tubulin at or near the vinca site (Sackett, 1995), it is likely that Rev is interacting with β -tubulin near the GTP binding pocket. The ability to depolymerize MTs resides in the N-terminal half of Rev as RTTs still form when the first 59 amino acids are mixed MTs polymerized with GTP. The ring formation by first 59 amino acids of Rev is inhibited with taxol stabilized MTs. Only short polymeric tubulin arcs form suggesting that the C-terminus is also important for RTT stabilization (Watts *et al.*, 2000).

A mechanism that explains Rev-tubulin interactions is suggested by the presence of a limited sequence similarity between the ARM of Rev and the catalytic motor domain of MCAK, a potent MT depolymerase and a member of Kinesin-13 group of proteins (Watts *et al.*, 2000; Figure 2). Rev residues Thr34, Arg39, and Arg42 are shared with MCAK residues Thr506, Arg511, and Arg514 and are thought to be surface exposed in both proteins. In Rev, they are important for RRE binding (Battiste *et al.*, 1996). The MCAK residues are vital for MT binding (Ogawa *et al.*, 2004). Hydrophobic residues Ile52, 55, and 59 that are important for interaction between helix-1 and 2 in Rev (Thomas *et al.*, 1997) are similar to corresponding residues in MCAK (Figure 2B) which reside in a helical structure (α 4 helix) and are

important for binding and depolymerization (Neiderstrasser *et al.*, 2001; Shipley *et al.*, 2004; Ogawa *et al.*, 2004). The α 4 helix is thought to bind the intradimer interface of the MT (Ogawa *et al.*, 2004).

Using the program HEX to model the interactions between tubulin heterodimers and the ARM peptide (PDB# 1ETF), the ARM is also proposed to bind tubulin heterodimers at the α and β -tubulin interface (Deacon Sweeney, pers. comm.). This prediction is consistent with the hypothesis that Rev binds the vinca (Maytansine) site of tubulin. This region of similarity also includes a part of helix-2 of the helix-loop-helix model proposed for the *N*-terminus for Rev.

Rev's ARM may mimic MCAK in several additional ways because MCAK has two positively charged regions in addition to the Rev-share sequences (Neiderstrasser *et al.*, 2002; Ogawa *et al.*, 2004). One of these regions is called the neck that lies adjacent to the motor domain. In motile kinesins, the neck confers motor processivity and directionality. In Kinesin-13s, the neck is rich in positively charged amino acids and is proposed to disrupt lateral interactions between adjacent protofilaments in a MT during depolymerization (Ogawa *et al.*, 2004). The second basic region is loop L8 that is important for MT binding (Niederstrasser *et al.*, 2002).

MCAK and MT depolymerization

MCAK is the best characterized member of Kinesin-13 family and plays an essential role in spindle formation and chromosomal movement during mitosis (Maney *et al.*, 1998, Ovechina *et al.*, 2002, Kline-Smith *et al.*, 2004). It consists of three separate domains: an *N*-terminal domain that targets kinetochores (Maney *et al.*, 1998, Wordeman *et al.*, 1999), a middle catalytic core preceded by the neck that is essential for MT depolymerization (Maney *et al.*, 2001, Ogawa *et al.*, 2004), and a *C*-terminal tail that regulates dimerization and ATPase activity (Maney *et al.*, 2001).

Maney *et al.* (2001) and Hertzner *et al.* (2006) have proposed that the MCAK monomer forms the minimal active unit but the dimer depolymerizes MTs three-fold more efficiently. Neiderstrasser *et al.* (2002) showed that MCAK acts on a single protofilament because it is able to depolymerize zinc polymerized tubulin, tubulin sheets made of anti-parallel protofilaments.

The positively charged neck of MCAK is proposed to act as electrostatic tether by interacting with negatively C-terminus of tubulin making MT depolymerization efficient (Maney *et al.*, 2001; Ovechina *et al.*, 2002). C-terminal E-hooks of MTs are also required although they are dispensable for binding MCAK (Neiderstrasser *et al.*, 2002). Limited digestion by protease subtilisin that cleaves E-hooks of tubulin shows that the C-terminal domain of β -tubulin is essential for microtubule depolymerization (Moores *et al.*, 2002; Neiderstrasser *et al.*, 2002). Moores *et al.* (2002) further proposed a model of depolymerization of microtubules wherein the motor core of pKinI (MCAK homolog in *Plasmodium falciparum*) binds to the ends of the microtubules with the subsequent release of ADP. The release of ADP leads to the binding of ATP that causes a bend in the tubulin dimer. This conformation change causes release of the dimer from the protofilament, followed by the dissociation of the motor core and tubulin with the subsequent hydrolysis of ATP (Moores *et al.*, 2002, Neiderstrasser *et al.*, 2002).

A comparison of the molecular structure of ADP bound form of Kif2C (murine ortholog of MCAK) and Kif1A (a motile kinesin) shows several differences in the following seven regions that are well conserved in Kinesin-13 group (Ogawa *et al.*, 2004). Six of these regions (L2, α 2-L5, L8- α 3-L9, L10, L11- α 4-L12, α 6) form a part of the characteristic, conserved motor domain of Kinesin-13 group of proteins,

the seventh being the neck region. Two of these regions, loop L8 and $\alpha 4$ helix, are shared with Rev (Ogawa *et al.*, 2004).

The Loop L2 in Kif2C has a characteristic 'KVD' motif (Lys293, Val294, Asp295) that forms a long, rigid finger like projection. This motif is appreciably smaller in Kif1A and mutation in this region reduces MT depolymerization significantly in Kif2C. The L5 loop is larger in Kif2C than Kif1A and is conserved in various members of Kinesin-13 family. The C-terminus of $\alpha 2$ helix is kinked at Pro384 in Kif2C. The longer L5 and kinked $\alpha 2b$ displace the main $\alpha 2$ helix by 10° in clockwise direction. This results in likewise rotation in following $\alpha 3$ helix that further results in change in positions of Loop L8 (lysine-arginine loop), which is proposed to serve as a sensor for regulation of ATP hydrolysis, and L9. Residues Asp502-Arg 506 at the end of L11 are wound up in $\alpha 4$ helix (the main MT binding region, Rev homology region), which make $\alpha 4$ helix longer in Kif2C and tilts it 5° compared to Kif1A.

X-ray crystallographic studies and *in silico* modeling suggests that the class specific residues at the end of $\alpha 4$ helix of the motor domain of Kif-2C binds the curved MT end and link the poly-glutamate tail of β -tubulin with α -tubulin (Ogawa *et al.*, 2004). The crosslinking of the poly-glutamate tails of the α - and β -tubulin stabilizes the curved structure of the intradimer interface and causes the initiation of ATP hydrolysis in Kif2C. Crosslinking also facilitates the insertion of KVD finger (absent in motile kinesins) in the interdimer interface and stabilizes the curved conformation of the protofilament. Mutating KVD impairs MCAK's ability to depolymerize MTs (Shipley *et al.*, 2004). The N-terminal region buries between protofilaments thus destabilizing the lateral interaction between of the protofilaments (Ogawa *et al.* (2004). Shipley *et al.* (2004) have also suggested that loop L11 and L2,

on the either side of motor core of pKinI form the anchor points on MTs. They have further suggested that mutating the KEC residues of the $\alpha 4$ helix inhibits MCAK's ability to depolymerize MTs (Shipley *et al.*, 2004). Mutating the 'E' residue of KEC in the $\alpha 4$ helix inhibited MCAK-MT binding (Ems-McClung *et al.*, 2007). Rev also has a conserved 'E' at position 57 similar to the 'E' residue of KEC, and this E57 in Rev also forms a part of helix-2 in Rev, suggesting that E57 might be playing an important role in MT interaction.

MODEL AND SPECIFIC AIMS

Any model that explains Rev-tubulin interactions must provide a mechanism that explains how Rev binds the MTs, how MT ends are peeled from the MT polymer, and ultimately how RTTs form.

Binding: There are several possible explanations explaining how Rev is able to bind MTs. The simplest explanation of electrostatic interactions between the ARM and E-hooks seems unlikely given the observation that RTT formation occurs even when the E-hooks are removed by subtilisin treatment (Watts *et al.*, 2000). The primary binding determinants must reside with the *N*-terminal 59 residues of Rev that can still form RTTs. These 59 residues includes the helix-loop-helix motif, the ARM and both multimerization domains as well as sequences shared with Kinesin-13 depolymerases (Watts *et al.*, 2000; Neiderstrasser *et al.*, 2002). Both the motor domain of Kinesin-13 proteins and ARM of Rev have been proposed to bind at the intradimer interface of MTs further supporting similar binding mechanism (Deacon Sweeney, per.comm.; Ogawa *et al.*, 2004). The shared sequences and secondary structures of Rev and motor domain of Kinesin-13 protein raise the possibility of ARM of Rev involved in MT binding.

Considering the similarities between Rev's ARM and Kinesin-13 proteins, Rev may be interacting with MTs in a manner similar to Kinesin-13-MT interaction. Rev's ARM may mimic the neck of Kinesin-13 proteins that is also rich in positively charged residues, regulates MT binding and is essential for MT depolymerization by disrupting of lateral interactions between protofilaments. It is also a possibility that Rev's ARM may be acting like MT binding Loop-8 of MCAK thus aiding in establishment of multiple contacts on MTs.

Depolymerization: MCAK has three distinct positively charged regions that help in MT depolymerization. The positively charged neck region helps in disrupting the lateral interactions between the MT protofilaments at MT ends (Ogawa *et al.*, 2004). Rev's ARM may behave similarly. The ARM may also act like the $\alpha 4$ helix of the catalytic domain of MCAK. The $\alpha 4$ helix is suggested to stabilize the curved conformation of MT ends (residues 268-270, 272). Since Rev shares the sequence similarity with this region of MCAK, there is a possibility that Rev's ARM may be acting in a similar manner. The similarities include but are not limited to Arg514 of MCAK, which is similar to Arg42 of ARM of Rev, which is mutated in M6. Another residue, Glu530, which when mutated in MCAK, inhibits its ability to depolymerize MT. Rev has a similar residue at position 57, suggesting that this amino acid may be participating in Rev's interaction with MTs. Similar possibilities exist for other shared residues mentioned in Figure 2B, supporting the possibility of similarity in mechanism for Rev-MT interactions. The third possibility is that Rev's ARM may be acting like the Lys-Arg loop of MCAK, involved in binding MTs (Ogawa *et al.*, 2004). During MT depolymerization when MCAK binds the MT ends, the neck region causes the disruption of lateral interaction between the protofilaments, loop L8 in the Lys-Arg loop region causes the hydrolysis of ATP while the $\alpha 4$ helix causes the stabilization of the curved conformation of MT ends. Rev might be acting in a similar way wherein the multimerizing Rev induces the curved conformation of MT ends and the ARM may act like neck or the $\alpha 4$ helix (the two positively charged regions of MCAK) to cause MT depolymerization.

RTT Formation: Watts *et al.* (2000) suggest that Rev causes MTs to peel outward forming RTTs. The formation of RTTs by outward peeling of MTs is consistent with tubulin lining the outside and Rev lining the inside of RTTs. During the formation the

RTT, it is possible that Rev binds the lattice, multimerizes on it and as it reaches the ends, it exerts an outward bending force needed to peel the protofilament from the MTs, like peeling a banana. Alternatively, it is possible that multiple Rev monomers bind the lattice first, multimerize until the MT end is reached, and MTs peel into rings. In either case, Rev multimerization provides multiple contacts on the surface of the MT and has the potential to generate a ripping/bending force required to make an RTT.

The formation of bilayered rings suggests that Rev may act upon two MT protofilaments at a time although it is formally possible that single layered rings form first that subsequently join. The second of the above two possibilities is supported by the fact that RTTs also form with tubulin heterodimers. The possibilities mentioned above support the idea that there might be a lateral interaction (dimerization) between Rev molecules in adjacent layers in a bilayered RTT.

Dimensional Considerations in an RTT: Watts *et al.* (2000) proposed a model of RTT in which 15 tubulin heterodimers lining the outside of the ring and 30 Rev monomers line the inside. The width of a tubulin monomer (4.3 nm) agrees well with the spacing of tubulin seen in the outer rim of RTT (Watts *et al.*, 2000). The inner rim of the ring had a spacing of 3.3 nm, consistent with the size of Rev dimers present in the Rev filaments (Watts *et al.* (1998). The above findings that the inner rim in an RTT has a spacing of 3.3 nm and that the width of a Rev dimer is 3.2 nm suggests that Rev may be binding tubulin heterodimers as a dimer (Figure 3). Since the diameter of the base of the ‘Top Hat’ is not sufficient (3.2 nm of Rev dimer as compared to 8 nm of a tubulin heterodimer) to interact with the adjacent Rev dimer (multimerization), the bound Rev dimer might introduce additional bends in the conformation of the heterodimer. There is also a possibility that the adjacent Rev dimer do not interact

with each other in the same layer (this could be due to limited ability of heterodimer to curve) but interact with the Rev dimer of the neighboring layer thus further aiding in formation of a bilayer (Figure 3).

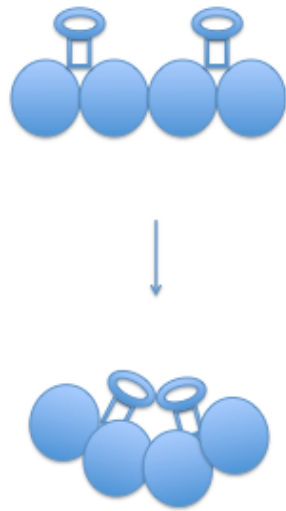
Specific Aims

Rev and Kinesin-13 proteins share more than a sequence similarity. Both depolymerize MTs (Watts *et al.*, 2000; Desai *et al.*, 1999). Both form rings when mixed with MTs (Watts *et al.*, 2000; Desai *et al.*, 1999; Moores *et al.*, 2002). Moreover, like Rev, MCAK homo-oligomerizes albeit it is only a dimer. Dimeric MCAK is a more potent depolymerase than the monomer by a factor of three (Hertzer *et al.*, 2006). Since Rev is also capable of multimerizing, **I tested the hypothesis that Rev multimerization is important for MT depolymerization.** To test this hypothesis, I compared and contrasted the ability of the multimerization defective mutant M4 form RTTs and depolymerize MTs to the wild-type protein using assays commonly used to monitor Kinesin-13 activity.

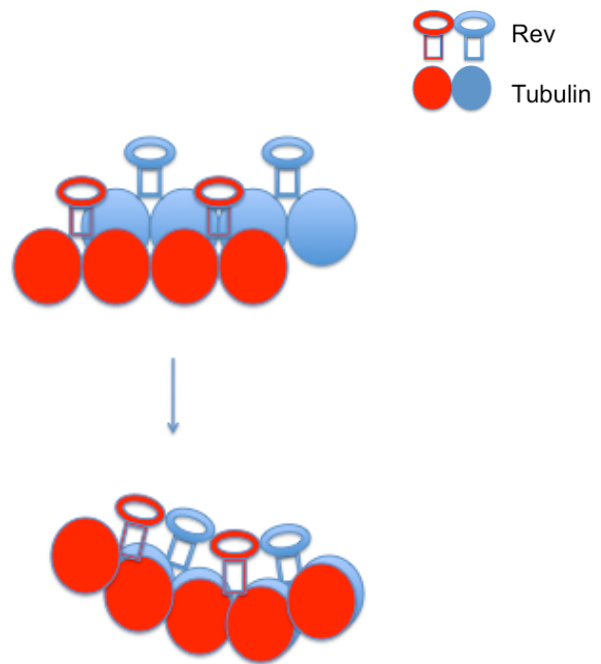
I have also tested the hypothesis that residues 41-44 in the ARM are important for Rev- MT interactions. Residues 41-44 are critical for RRE binding and comprise a part of Rev-Kinesin-13 shared region. For these experiments, I will compare the M6 mutant with wild-type Rev for its ability to depolymerize MTs and form RTTs. Mutation in M6 includes Arg42 that is similar to critical residue Arg514 that lies in MT binding region of MCAK. Inability of M6 to interact with tubulin will show that residues 41-44 form a minimum region in Rev responsible for binding and depolymerization. Conversely, formation of any kind of complex, determined by combination of sedimentation assays, gel filtration assays and electron microscopy, will suggest the role of other shared residues in ARM. Taken together, I

Figure 3: Proposed spatial arrangement of Rev and tubulin in RTT. **A)** Rev binds the intradimer interface of tubulin as a dimer. The interaction between adjacent Rev dimer (top hat) on the tubulin intradimer interface creates a force required for introducing the curved conformation in the terminal heterodimer in a MT and cause depolymerization. **B)** Alternatively, Rev bound to one tubulin heterodimer may multimerize with a Rev dimer bound to diagonally opposite heterodimer on the neighboring protofilament, together generating the force required for MT peeling and RTT formation.

A



B



have tested the above hypothesis by performing experiments that test whether residues 23, 25 and 26 are required for binding tubulin heterodimers and forming RTTs. I have also performed experiments that test the importance of these residues in MT depolymerization.

MATERIALS AND METHODS

Rev purification

Rev and mutants: The plasmid pET11D-Rev encoding wild-type Rev was kindly provided by Dr. Blanco (Blanco *et al.*, 2001). Point mutations were introduced in the wild-type gene using the Quikchange Site Directed Mutagenesis Kit (Stratagene Inc., CA), using primers listed in Appendix A and confirmed by double stranded sequencing (Davis Sequencing, Davis CA). The wild-type and mutant plasmids were perpetuated in *E.coli* DH5 α cells and stored in 15% glycerol stocks at -80°C.

To express Rev, BL21(DE3) cells were transformed with Rev expression plasmids that were grown and selected on Luria-Bertini (LB) agar plates supplemented with 50 μ g/ml ampicillin. Large scale 1 L LB/amp cultures were inoculated with 50 ml overnight cultures initiated from a single colony from a plate stock. All cultures were grown at 37°C with 250 rpm shaking. Expression of Rev was induced with the addition of 1 mM IPTG when cultures exhibited exponential growth (OD₆₀₀≈0.6). Two hours later, cells were harvested by centrifugation at 5,000 x g for 10 min. Cell pellets were frozen at -80°C until Rev was purified.

Purification: Cell pellets were resuspended in a buffer A (50mM Tris-HCl pH 8.0, 1 mM DTT, 1 mM EDTA, and 5 mM PMSF) supplemented with 400 mM NaCl and lysed by sonication, three 30 s pulses at 10 w. Sonicates were frozen at -80°C for five minutes, quickly thawed and sonicated as before. Sonicates were clarified by centrifugation at 12,000 x g for 10 min in a SS34 rotor (Sorvall, CA) and FPLC purified using the methods of Karn *et al.* (1995). Briefly, lysates were fractionated using a Q-Sepharose column (GE Life Sciences, NJ). The column was washed with buffer A plus 400 mM NaCl and Rev-RNA complexes were eluted with buffer B (Buffer A plus 800 mM NaCl). The eluate was collected and fractionated using a

Heparin-Sepharose column (GE life sciences, NJ). The column was washed with five column volumes of buffer B followed by five column volumes of buffer C (Buffer A plus 1 M NaCl). Rev was eluted with buffer D (Buffer A plus 2 M NaCl). The presence of Rev was confirmed by immunodetection using Rev specific antibodies and the purity confirmed by SDS-PAGE and coomassie brilliant blue staining. Figure 4 shows representative results of purification.

Rev obtained from heparin-sepharose chromatography was monomeric owing to the high salt concentration (2 M) that must be diluted before mixed with MTs. MTs depolymerize in 500 mM salt (Mendez, 1998). However, because Rev aggregates under low salt conditions (Wingfield *et al.*, 1991, Watts *et al.*, 2000), purified Rev was denatured and refolded into filaments using the methods of Watts *et al.* (1998) and concentrated by ultrafiltration (Centricon-30) or ultracentrifugation (320,000 x g, 4 hours, in a TLA100 rotor). Protein concentrations were determined spectrophotometrically ($\epsilon=8.31 \text{ mM}^{-1}\text{cm}^{-1}$) and by densitometric scanning of coomassie stained proteins relative to BSA standards resolved by SDS-PAGE using ImageJ (Rasband, 1997-2007).

Alternatively, wild-type and M4 were also purified with an alternative modified protocol derived from Wingfield *et al.* (1991). In the modified protocol the DEAE-sepharose and Fast S columns are substituted with Q-sepharose and heparin-sepharose columns, respectively. Rev-expressing cell pellets from 1 L cultures of *E. coli* were sonicated as above in lysis buffer (0.1 M Tris-HCl, pH 8.0, 5 mM DTT, 5mM PMSF, 5mM benzamidine and 5 mM EDTA). Sonicates were subjected to 10,000 x g centrifugation for 10 min. The supernatant was collected and further subjected to a spin at 60,000 x g for one hour. The supernatant was collected and diluted 3 fold in buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM sodium azide and

applied over Q-sepharose column. Rev eluted in the flow-through fractions, which were collected and supplemented adjusted to 6.5 using phosphoric acid. The Rev containing solution was then applied over with solid urea to a final concentration of 6 M. The resultant solution was diluted in buffer E (0.02 M sodium phosphate, pH 6.5, 1 mM EDTA, 1mM DTT and 2 M urea) and pH heparin-sepharose column. The column was then washed with buffer E which was followed by application of a five column volume gradient of NaCl (0-1 M) in buffer E. Rev elutes off at 0.5 M NaCl which was then subjected to refolding and concentration determination as described above.

M6 purification: Rev purified using the above methods relies on the ability of Rev to bind cellular RNA which then binds the Q-sepharose column. This complex is then dissociated by high salt and Rev is purified exploiting its affinity for heparin. However, this procedure does not work well for M6 that has reduced affinities for RNA and heparin. To purify M6, I used a modified protocol derived from Wingfield *et al.* (1991). Bacterial cell lysates clarified by 60,000 x g centrifugation were introduced over Q-Sepharose column under no salt conditions (0.1 M Tris-HCl, pH 8.0, 5 mM DTT, 5mM PMSF, 5mM benzamidine and 5 mM EDTA). The absence of salt allows for limited Rev aggregation, which elutes off separating it from nucleic acids (bound to the column). Rev, present in the flow-through, is denatured in 6 M urea, and subjected to refolding as described above in the Rev purification section. Since M6 cannot be completely purified, BL21(DE3) *E.coli* whole cell extracts were used as a control.

Tubulin Purification

Tubulin was purified from freshly procured bovine brains according to the methods of Walczak and Desai, 2001. Briefly the meninges are stripped and brains

were homogenized in Pipes buffer (PB) (0.1 M K-Pipes pH 6.8, 0.5 mM MgCl₂, 2 mM EGTA, 0.1 mM EDTA, 0.1 % β -mercaptoethanol, 1 mM ATP). The lysate was centrifuged at 33,000 x g for 2 hr at 4°C using a GS3 rotor (Sorvall). The supernatant was collected and supplemented with 1.5mM ATP, 0.1 mM GTP, 3.5 mM MgCl₂ and 50% glycerol (final concentrations) and incubated at 37°C, 1 hr. The extract was subjected to centrifugation at 33,000 x g, 165 min, 35°C. The pellet was recovered, resuspended in cold PB, homogenized in presence of additional 1 mM ATP and 0.1% β -mercaptoethanol, and incubated at 4°C, 40 min. The homogenate was subjected to centrifugation at 190,000 x g, 30 min, 4°C, in a Beckman type 50.2 Ti rotor and the supernatant containing tubulin heterodimers was recovered. The supernatant was supplemented with warm 50% glycerol, 0.5 mM GTP and 4 mM MgCl₂, at 37°C for 1 hr. The extract was centrifuged at 240,000 x g 30 min centrifugation at 35°C, in a Beckman type 50.2 Ti rotor. The pellet was resuspend in column buffer (CB) (50 mM K-Pipes, pH 6.8; 1 mM EGTA; 0.2 mM MgCl₂). The pellet was dounce homogenized and incubated at 4°C for 40 min to depolymerize MTs. The supernatant was recovered after centrifugation at 190,000 x g, 30 min, 4°C and applied over a 200 ml phosphocellulose (PC) column equilibrated in CB. Tubulin present in the flow-through fractions was collected, pooled and flash frozen in 7 ml aliquots in liquid nitrogen (Figure 5). This tubulin was called PC tubulin. A small fraction was used to determine the concentration using spectrophotometric (O.D. 280 nm, ϵ of tubulin 115,000 M⁻¹cm⁻¹) and densitometric methods using ImageJ and bovine gamma globulin as a standard.

Sedimentation assays

Purified tubulin heterodimers were diluted to 3 μ M in MEM (100 mM MES, 1 mM EGTA, 2 mM MgCl₂, pH 6.9) on ice for 10 min and clarified by 360,000 x g

centrifugation using a TLS optima centrifuge. In some experiments, the supernatant was supplemented with 50 μ M colchicine (Sigma, St. Louis, MO) and diluted to the desired concentration in MEM. In other experiments, the supernatant was supplemented with GMPcPP to 1 mM final concentration and incubated at 37°C for 30 min. Polymerized microtubules were recovered by 360,000 x g, 5 min centrifugation at 37°C and diluted to twice the desired final concentration in MEM. Rev was diluted in MES (100 mM MES, 2 mM MgCl₂, pH 6.9) to twice the desired final concentration (~10 μ M) immediately before use. In most instances, MgCl₂ was added ensuring the final concentration was 1-2 mM above the citrate concentration. Reactions were initiated by mixing equal volumes of tubulin or microtubules with Rev for prescribed times at room temperature. Samples were subjected to 5 min, 360,000 x g centrifugation. Pellet and supernatant fractions were recovered and the proteins present in each were resolved by SDS-PAGE (Desai and Walczak, 2001). Gels were stained with coomassie brilliant blue and the amount of tubulin present in each fraction was quantified using ImageJ (Rasband, 1997-2008). In some instances, proteins were transferred to nitrocellulose membranes at 50 mV for 1.5 hr in transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, 20% ethanol). Rev and tubulin were detected by immunoblotting using protein specific antibodies (sheep anti-Rev, US Biologicals, Swampscott, MA; DM1a, Sigma, St. Louis, MO).

Transmission electron microscopy (TEM)

Aliquots (5 μ l) of mixtures described above were spotted onto a carbon coated mica grid (Ted Pella, CA). After 1 min, excess sample was wicked away and the sample was fixed with 1% uranyl acetate for 20 s. The sample was stained with 1% uranyl acetate for 1 min, air dried and visualized with a Philips EM 208S electron microscope at 56000x magnification.

Gel filtration chromatography (superdex-200)

Mixtures of Rev and colchicine-treated tubulin were resolved on a Superdex-200 gel filtration column equilibrated with 150 mM sodium phosphate (pH 7.2) and 50 mM sodium chloride. Aliquots of collected fractions were resolved by SDS-PAGE, and transferred onto nitrocellulose membranes. Rev and tubulin were detected using protein specific antibodies.

Statistical Analysis

Statistical analysis for data obtained from sedimentation assays involving tubulin heterodimers and Rev (in presence and absence of Mg^{++}) was performed using one-way ANOVA. When required, Tukey's HSD posthoc testing was used.

Regression model, Bonferroni corrections and students T-test analyzed data obtained from sedimentation assays.

RESULTS

Purification of tubulin, wild-type Rev, M4 and M6

Wild-type Rev, M4 and M6 were over-expressed in BL21(DE3) cells and purified using FPLC, as described in “Materials and Methods”. Presence of Rev was confirmed using Rev specific antibodies. Whereas purified wild-type Rev and M4 were largely devoid of impurities, M6 was not comparably purified because it did not bind the heparin column (Figure 4). Despite the impurities, TEM analysis demonstrates that wild-type Rev and M6 form similar ≈ 13 nm filaments, consistent with the published work (Figure 5) (Watts *et al.*, 1998). There were no signs of filament bundling or aggregation. On the contrary, M4 did not form filaments. Large, irregular shaped, high molecular weight complexes were present, consistent with the idea that the M4 mutation inhibits Rev-Rev interactions (Figure 5). Tubulin was purified from bovine brains, as described in “Materials and Methods” (Figure 6).

Measuring interactions between wild-type Rev and tubulin heterodimers

Sedimentation assays were performed to determine whether Rev binds tubulin and forms high molecular weight complexes. These assays are routinely used to characterize MCAK-MT interactions (Desai and Walczak, 2001). To compare the ability of Rev to interact with tubulin and form high molecular weight complexes, different concentrations of Rev were incubated with tubulin for 10 minutes and centrifuged at 360,000 x g. In the absence of Rev, tubulin heterodimers fractionate into the supernatant following centrifugation. However, with the addition of different concentrations of wild-type Rev in the presence of Mg^{++} , significant amounts of tubulin fractionates into the pellet (one-way ANOVA; $p=0.013$). Posthoc test using Tukey’s HSD demonstrates that significant amount of tubulin fractionates in the pellet

Figure 4: Rev Purification. Representative results of proteins resolved by SDS-PAGE. Rev expressed in BL21(DE3) cells were lysed, fractionated (bound and eluted) using Q-Sepharose (**A**) and heparin-sepharose (**B**). Purified wild-type Rev, M4 and M6 used in the experiments are shown in **C**, **D** and **E** respectively. Gels are stained with coomassie brilliant blue. The markers in A and B correpond to 180 (top), 115, 82, 64, 49, 37, 26, 19, 15 and 6 kDa (bottom). The markers in C, D and E correpond to 170 (top), 135, 100, 72, 55, 42, 33, 24, 17 and 11 kDa (bottom).

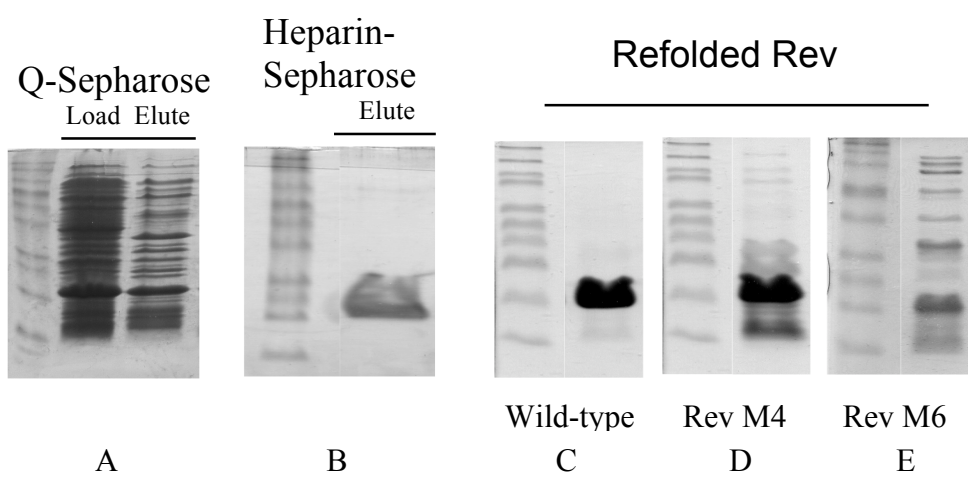
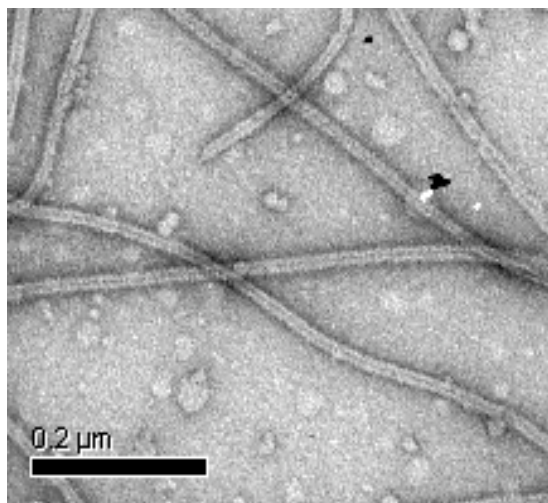
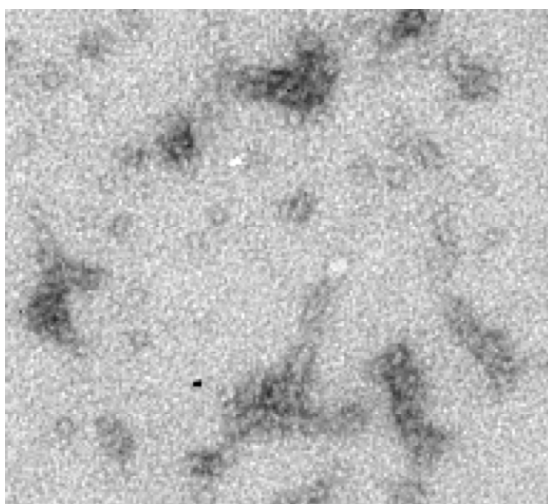


Figure 5: Electron micrographs of purified Rev and mutants: **A.** purified and refolded wild-type Rev, **B.** M4, and **C.** M6. Magnification bar correspond to 200nm.

A.



B.



C.

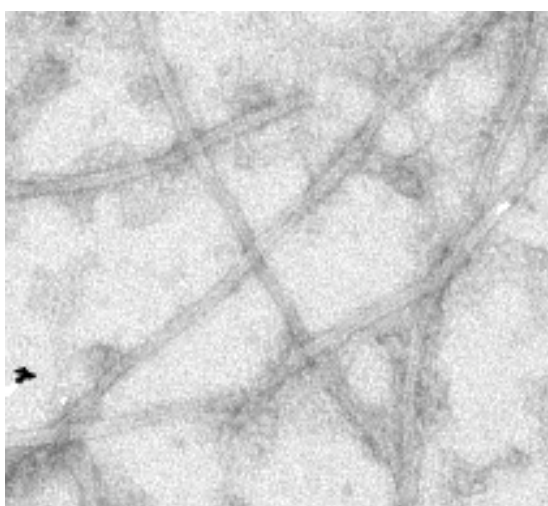
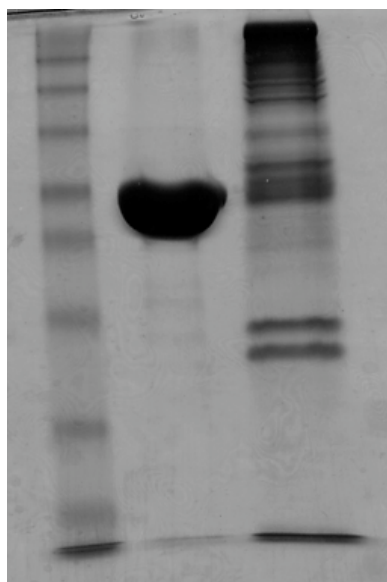


Figure 6: Tubulin Purification. Tubulin was purified as described in materials and methods. A representative coomassie stained gel, depicting tubulin (**A**) separated from MAPS (**B**) after phosphocellulose chromatography.



A B

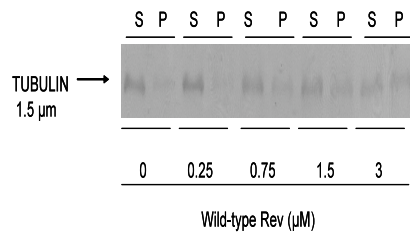
at 1:1 Rev-tubulin heterodimer ratios ($p < 0.05$). The formation of high-molecular weight complexes is consistent with the formation of RTTs (Figure 7). Although not statistically significant, more tubulin is detected in the pellet fractions at lower concentrations than is seen with the wild-type protein (Figure 7). Sedimentation assays further show that compared to no Rev control, product formation takes place at 1:1 wild-type Rev monomer/tubulin heterodimer molar ratio in the absence of Mg^{++} (one-way ANOVA, $p < 0.0001$) (Figure 7). However, high molecular weight complexes were absent below Rev monomer-tubulin ratio of 1:1 (Figure 7).

The formation of pelletable complex in the absence of Mg^{++} is contradicts the results obtained by Watts *et al.* (2000). To investigate the formation of pelletable products in the absence of Mg^{++} and to determine the sizes of products formed in the absence and presence of Mg^{++} , S200 size exclusion chromatography was performed. Size exclusion chromatography provides the advantage of separating high molecular weight complexes based on size, with larger complexes eluting out faster whereas the smaller ones have to pass through the pores of the chromatography beads. In the absence of Rev, colchicine-treated tubulin elutes in fractions 15 corresponding to the size of 142 kDa (Figure 8). 500 μ l fractions were collected during gel filtration chromatography which may explain the calculated size of tubulin heterodimer to be 142 kDa and not 110 kDa. It is a possibility that collection of smaller fractions may provide a better approximation of the size of the tubulin heterodimer.

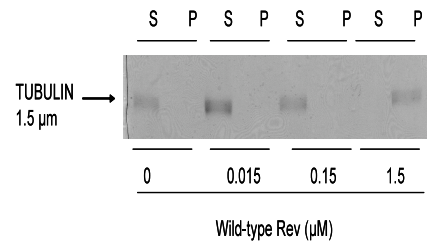
The peak in the chromatogram is broad suggesting the presence of oligomers of tubulin in addition to heterodimers. When Rev-tubulin heterodimer mixtures in 1:1 molar ratio were resolved in the absence of Mg^{++} , Rev and tubulin eluted in fractions corresponding to ≈ 142 kDa (Figure 9). The formation of similar-sized complexes in the presence and absence of Rev suggest that the experiments in the absence of Mg^{++}

Figure 7: Wild-type Rev forms high molecular weight complexes when mixed with tubulin heterodimers. **A.** Coomassie stained gel showing tubulin present in the supernatant (S) and pellet fractions (P) after tubulin heterodimers are mixed with different concentrations of wild-type Rev in the presence of Mg^{++} . **B.** Graphical representation of the quantification of tubulin heterodimers in the (S) fraction after incubation with wild-type Rev, in the presence of Mg^{++} . **C.** Coomassie stained gel showing tubulin present in the (S) and (P) fractions after tubulin heterodimers are mixed with different concentrations of wild-type Rev, in the absence of Mg^{++} . **D.** Graphical representation of the quantification of tubulin heterodimers present in the (S) fraction after incubation with wild-type Rev, in the absence of Mg^{++} . Black star corresponds to $p < 0.05$ (one-way ANOVA, $n=15$; PostHoc test using Tukey's HSD); Red stars corresponds to $p < 0.0001$ (one-way ANOVA, $n=12$; PostHoc test using Tukey's HSD). Error bars correspond to standard deviations.

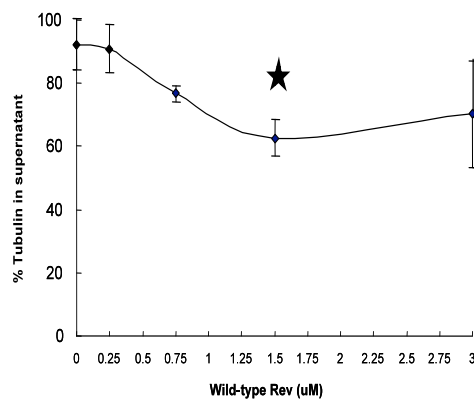
A.



C.



B.



D.

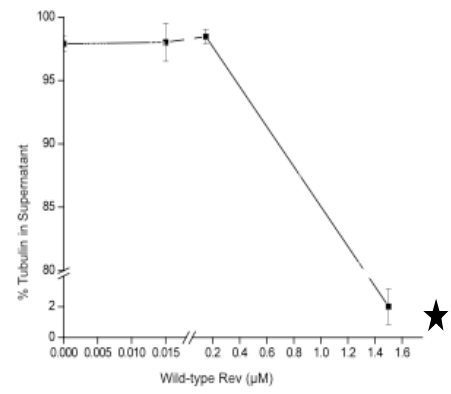
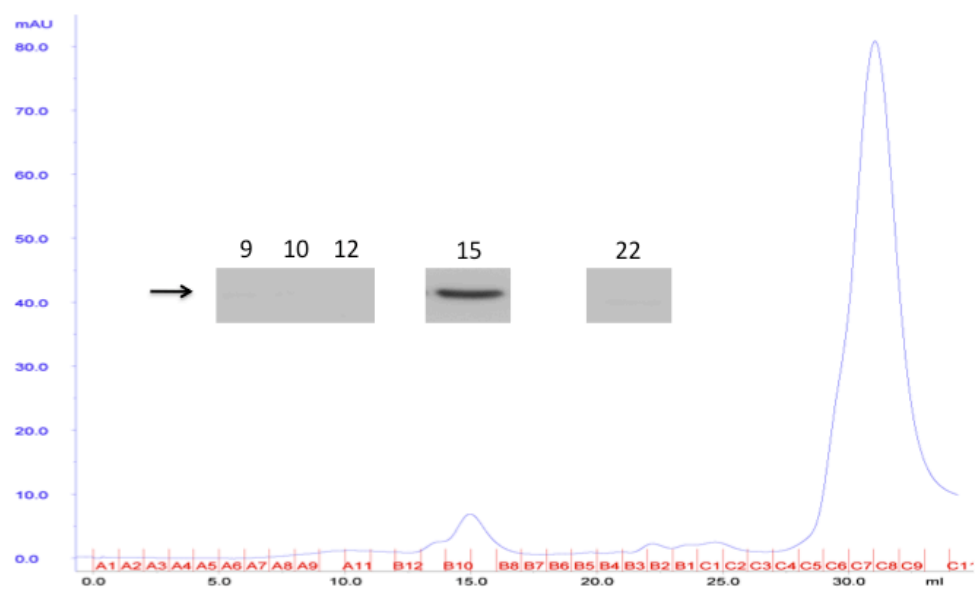
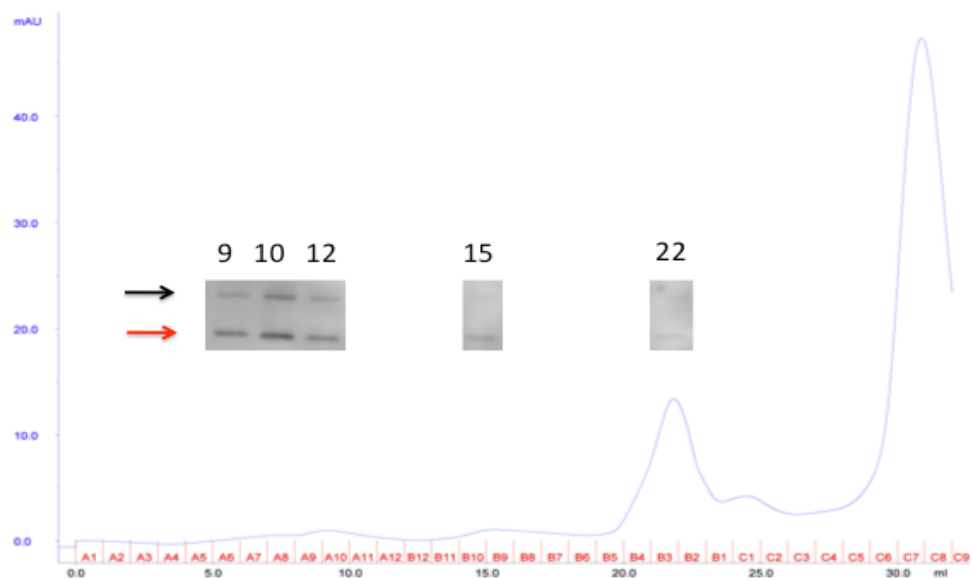


Figure 8: Gel filtration of reactions involving Rev and its mutants with tubulin in the presence of Mg^{++} . Representative chromatograms from S200 gel filtration chromatography (presence of Mg^{++}) and immunoblots detecting tubulin (black arrow) and Rev (red arrow) present in the designated fractions for **A.** Colchicine treated tubulin alone, **B.** Wild-type Rev and colchicine treated tubulin, **C.** M4 and colchicine treated tubulin, and **D.** M6 and colchicine treated tubulin. Representative chromatograms from S200 gel filtration chromatography of gel filtration standards: **E)** Dextran Blue (black arrow, 2 MDa), **F)** Molecular weight standard proteins showing elution peaks for Ferritin (F, 440 kDa), Aldolase (A, 158 kDa), Conalbumin (C, 75 kDa) and Ribonuclease A (R, 13.7 kDa).

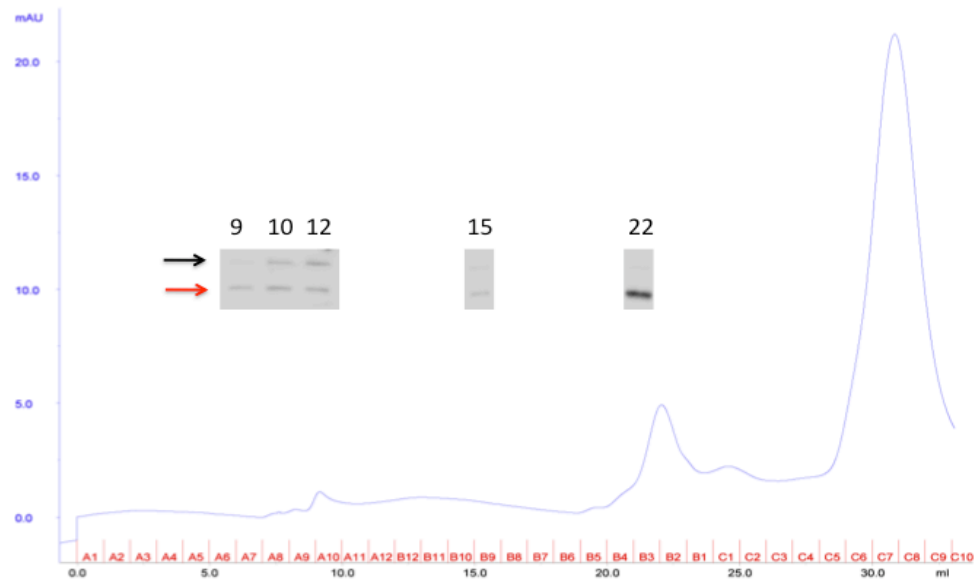
A.



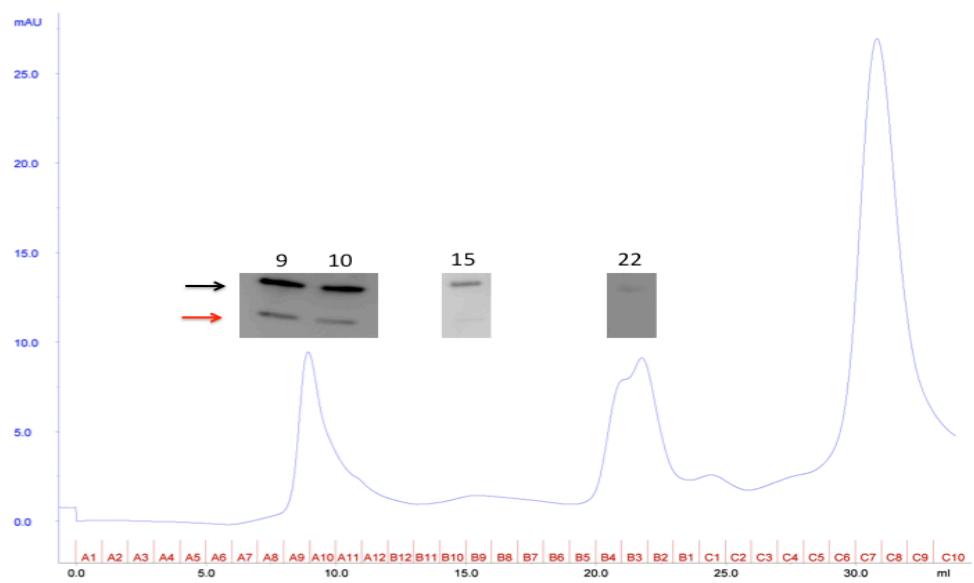
B.



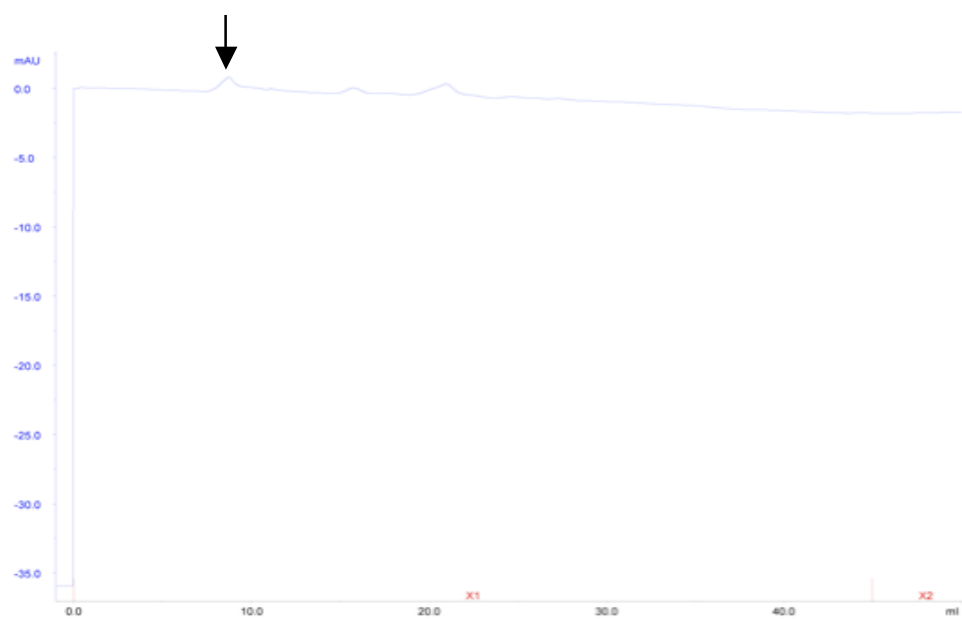
C.



D.



E.



F.

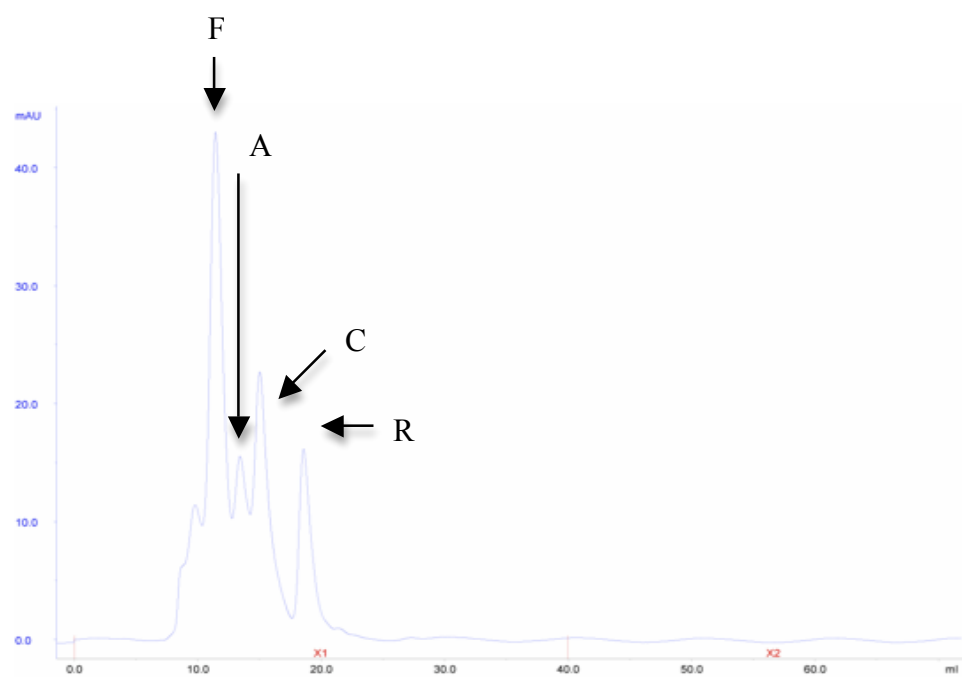
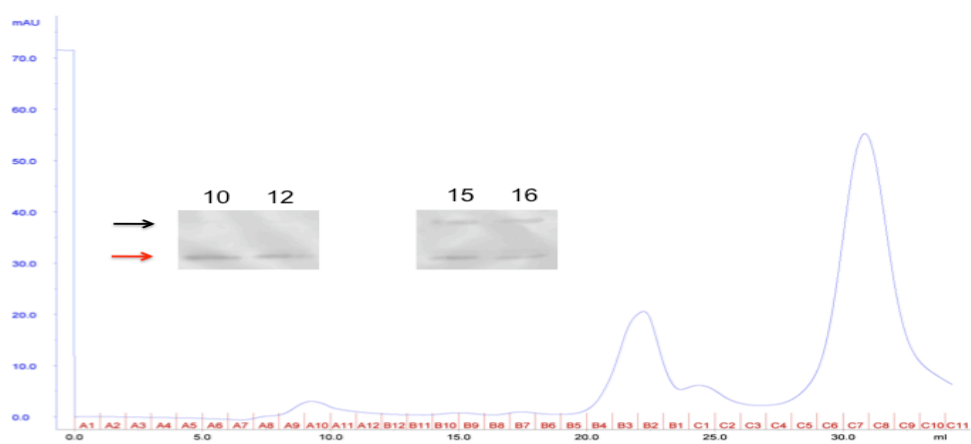
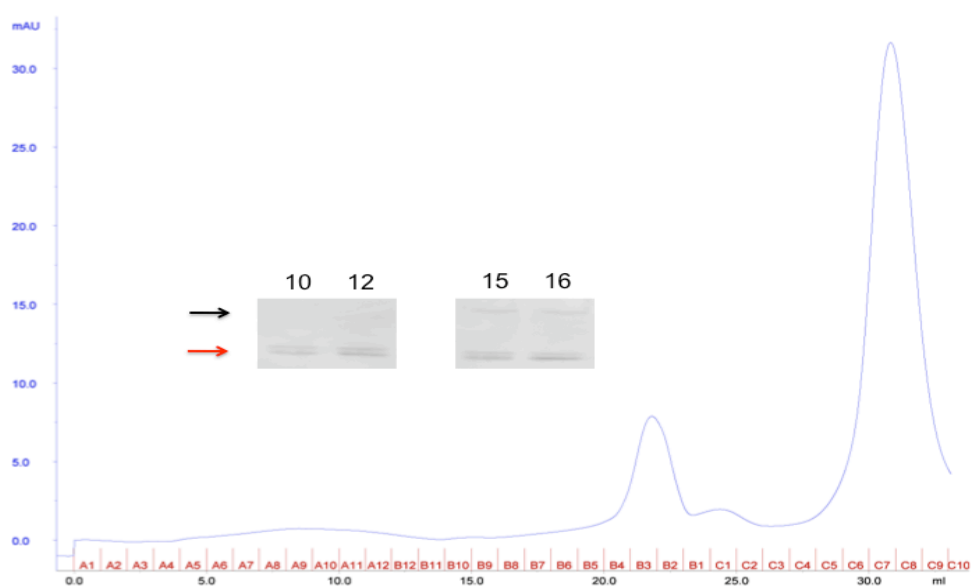


Figure 9: Gel filtration of reactions involving Rev and its mutants with tubulin in the absence of MgCl_2 . Representative chromatograms from S200 gel filtration chromatography (absence of Mg^{++}) and immunoblot for Tubulin (black arrow) and Rev (red arrow) in corresponding fractions for **A.** Wild-type Rev and colchicine treated tubulin, and **B.** M4 and colchicine treated tubulin, **C.** M6 and colchicine treated tubulin.

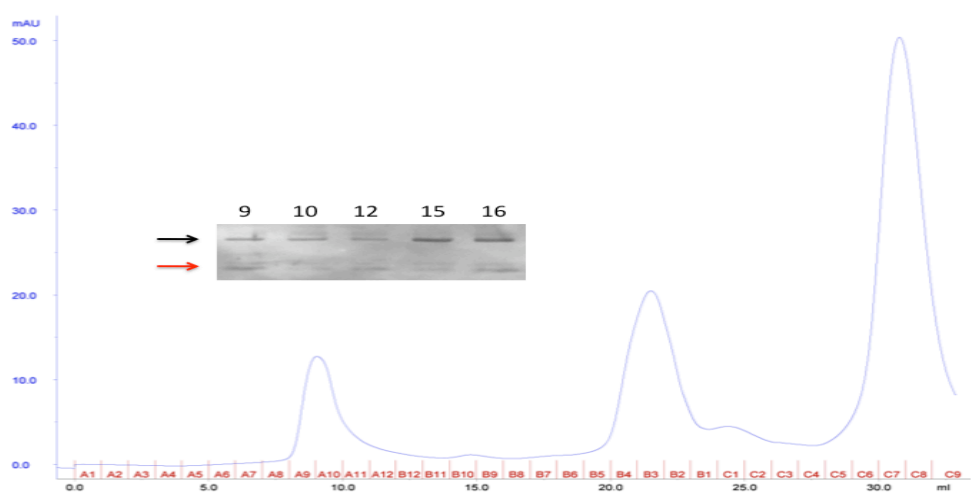
A.



B.



C.



need to be performed using gel-filtration column that will facilitate better resolution of complexes. The absence of Rev-tubulin complex in the void volume however suggests that the pelletable complex detected in the sedimentation assays in the absence of Mg^{++} may be the rings that are unstable and hence not detected during the S200 chromatography.

Chromatography of Rev:tubulin (1:1 molar ratio) mixtures supplemented with Mg^{++} show a significant amount of Rev and tubulin elute in the void volume (fractions 9 and 10), corresponding to complexes with sizes greater than $M_r \approx 500$ kDa (Figure 8). The presence of tubulin and Rev in the void volume suggests the formation of high molecular weight complex that is consistent with presence of RTT.

To determine whether the materials in the pellets are RTTs, aliquots of Rev-tubulin mixtures were spotted on a carbon coated mica grid, stained with 1% uranyl acetate, dried and visualized using TEM. RTTs formed at 1:1 molar ratio of Rev monomer and tubulin heterodimer (Figure 10). Consistent with the diameter of RTTs observed by Watts *et al.* (2000), the majority of RTTs observed during this study were ≈ 40 nm in diameter.

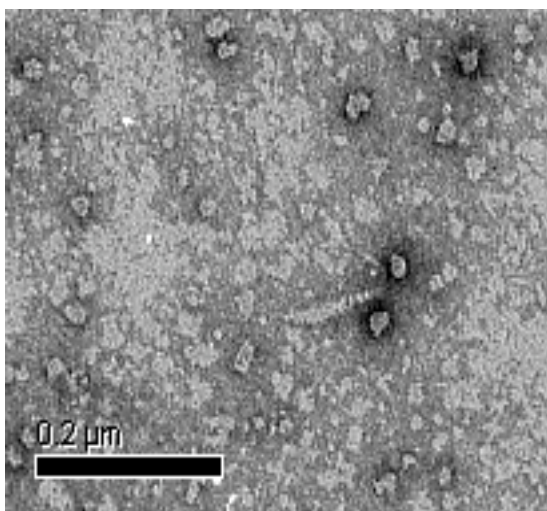
Taken together these data confirm the results of Watts *et al.* (2000), validate the sedimentation assays and show that Rev used in the study is functional.

Measuring interactions between M4 and tubulin heterodimers

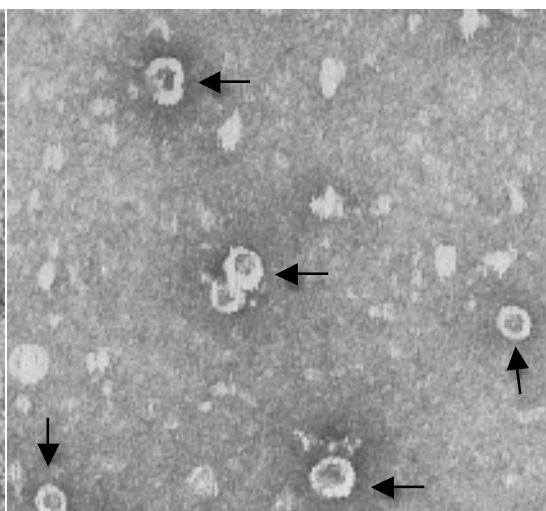
Sedimentation assays show that M4 bound tubulin heterodimers significantly in the presence of Mg^{++} in a concentration dependent manner (one-way ANOVA $p < 0.0078$) (Figure 11). Post hoc tests performed using Tukey's HSD demonstrated that complex formation was significant at concentrations $\geq 1.5 \mu M$ ($p < 0.05$). Although M4 appears to sediment more tubulin than the wild-type protein, these differences are

Figure 10: Electron micrographs of mixtures of tubulin heterodimers in Rev buffer (A), with wild-type Rev (B), M4 (C) and M6 (D): Black, yellow and red arrows point to complexes formed with wild-type Rev, M4, and M6, respectively. Magnification bar correspond to 200 nm.

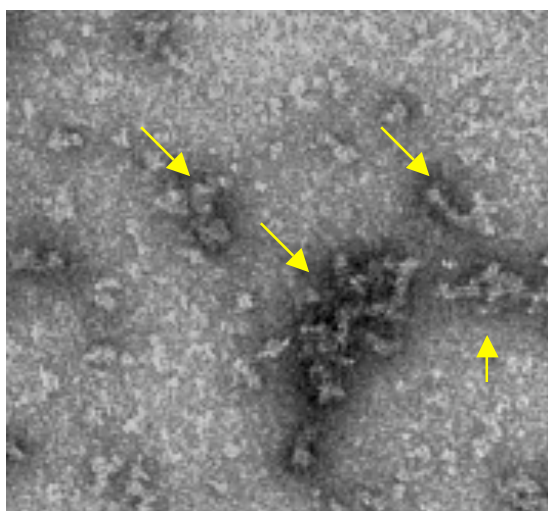
A



B



C



D

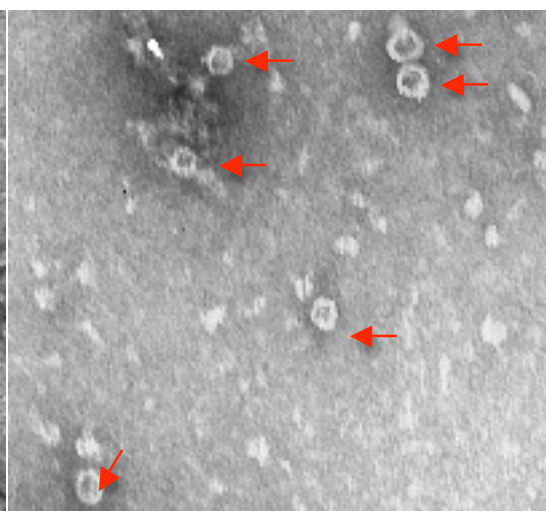
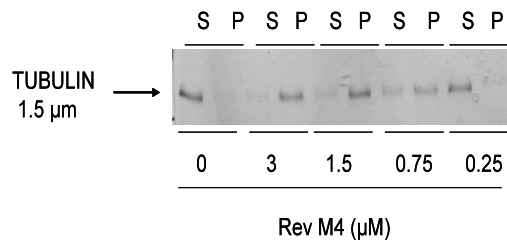
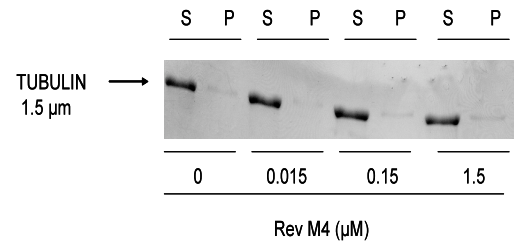


Figure 11: Sedimentation assays with M4 and tubulin. **A.** Coomassie stained gel showing tubulin concentration in the supernatant (S) and pellet fraction (P) when tubulin heterodimers are incubated with varying concentration of M4, in the presence of Mg^{++} . **B.** Graphical representation of the quantification of tubulin heterodimers in the (S) fraction after incubation with M4 in the presence of Mg^{++} . **C.** Coomassie stained gel showing tubulin concentration in the (S) and (P) fraction when tubulin heterodimers are incubated with varying concentration of M4, in the absence of Mg^{++} . **D.** Graphical representation of the quantification of tubulin heterodimers present in the (S) fraction after incubation with M4 in the absence of Mg^{++} . Black stars correspond to $p=0.05$ (one-way anova, $n=15$; Posthoc test using Tukey's HSD). Error bars correspond to standard deviations.

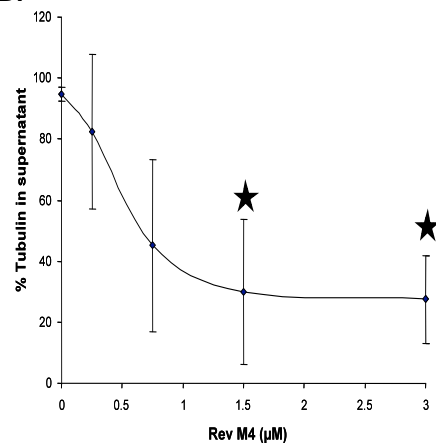
A.



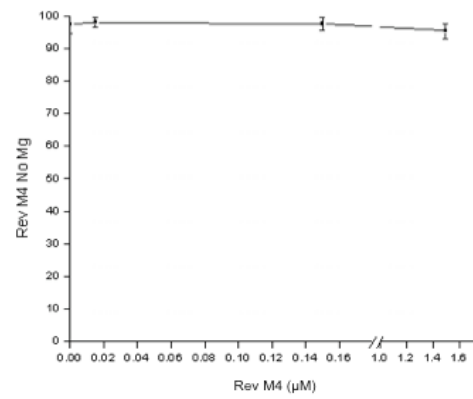
C.



B.



D.



not statistically significant (one-way ANOVA; $p=0.8$). M4 did not form high molecular weight complexes in the absence of Mg^{++} (one-way ANOVA; $p=0.5$) (Figure 11).

S200 gel filtration chromatography was performed to investigate the sizes of the complex formed by M4. The results in the presence of Mg^{++} show that similar to wild-type Rev, RevM4 formed high molecular weight complexes that elute out in the void volume and contain both Rev and tubulin (Figure 8). Elution of tubulin and Rev together in fractions 15 and 16 suggests that only low molecular weight complexes (consisting of both Rev and tubulin) are observed in the absence of Mg^{++} (Figure 9).

TEM analysis in the presence of Mg^{++} shows that M4 does not form RTTs. Instead heterogeneous clusters are seen (Figure 10). Sedimentation assays conducted in parallel with TEM show that almost 94% of tubulin shift from the supernatant to pellet fraction after incubation with M4 (Figure 12).

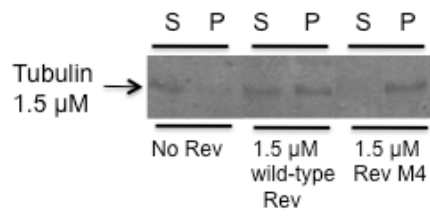
These data collectively argue that the M4 mutation does not block tubulin binding or the formation of high molecular weight complex formation. However, it does block RTT formation.

Measuring interactions between wild-type Rev and MTs

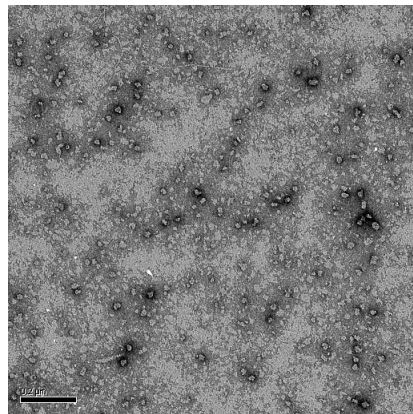
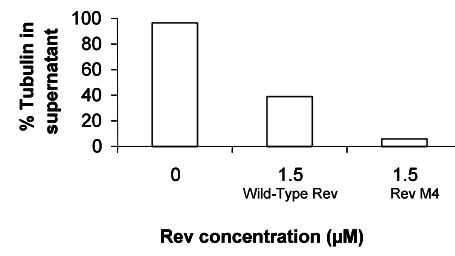
Previous studies suggest that wild-type Rev depolymerizes GMPCPP polymerized MTs into spirals (Watts *et al.*, 2000). GMPCPP is a slowly hydrolysable analog of GTP and prevents MT from spontaneously depolymerizing during the course of experiments. To compare the abilities of wild-type Rev and M4 to depolymerize stabilized MTs, sedimentation assays were conducted. In the absence of Rev, stabilized MTs sediment in the pellet fraction after high-speed centrifugation. Rev's effect on MTs can be determined by observing release of tubulin heterodimers

Figure 12: Sedimentation assays corresponding TEM of wild-type Rev, M4 with tubulin **A.** Coomassie stained gel showing tubulin present in supernatant (S) and pellet (P) fractions in absence of Rev and when tubulin is incubated with wild-type Rev and M4, in the presence of Mg^{++} . **B.** Graphical representation of tubulin in supernatant fraction after it is treated with wild-type Rev and M4. **C, D, and E.** Representative electron micrographs of tubulin alone (**C**), RTT formed in presence of wild-type Rev (**D**) and high molecular weight complex in presence of M4 (**E**).

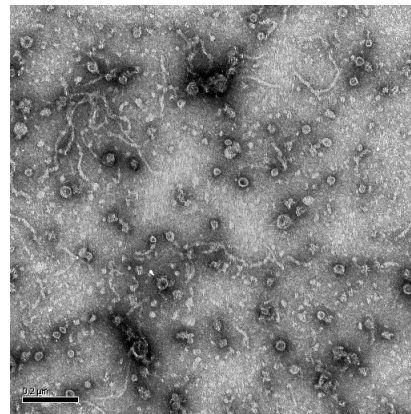
A.



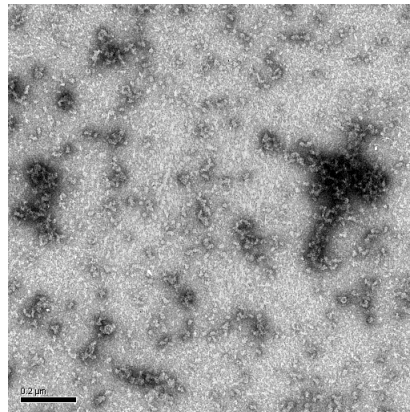
B.



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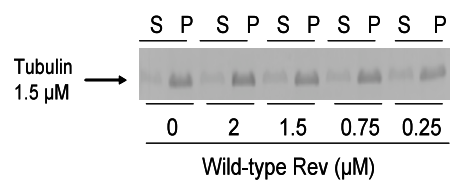
E.

in the supernatant fraction. However, measuring depolymerization is complicated by the fact that any tubulin released from MTs may bind Rev and form RTTs (Figure 10). Since RTTs also fractionate into the pellet, the true amount of tubulin present in the supernatant will be underestimated. However, sedimentation assays can be used to qualitatively demonstrate MT depolymerization stimulated by Rev addition. Sedimentation assays reveal that wild-type Rev releases significant amounts of tubulin heterodimers from MTs both in the presence and absence of Mg^{++} (linear regression and Bonferroni correction; $p < 0.04$ and $p < 0.0001$, respectively) (Figure 13). Statistical analysis also shows that depolymerization is more significant in the absence of Mg^{++} than in the presence of Mg^{++} (linear regression and Bonferroni correction; $p < 0.01$). More depolymerization in the absence of Mg^{++} is consistent with the finding that the presence of Mg^{++} promotes high molecular weight complex formation that may prevent detection of released tubulin during sedimentation assays with Rev and MTs. Even at Rev:tubulin heterodimer stoichiometries of $>1:1$, roughly 50% of the tubulin remained in the pellet suggesting either that Rev is unable to depolymerize all the MTs in the sample or that some of the depolymerization products are forming RTTs or other high molecular weight complexes.

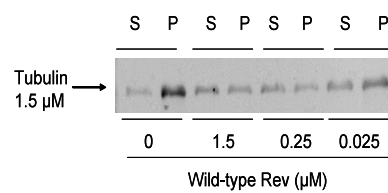
TEM of Rev-MT mixtures show that MT and RTTs are both present in these samples (Figure 14). MTs are shorter ($\approx 16.4 \mu m$ compared to control MTs that were $\approx 33 \mu m$) (one-way ANOVA; $p < 0.0001$) and their ends appeared disorganized, consistent with microtubule depolymerization. Their edges appear darkly stained and fuzzy suggesting that Rev is coating the lattice. Measurement of fluorescently labeled microtubules confirms there are two-fold fewer and $\approx 50\%$ shorter microtubules -

Figure 13: Sedimentation Assay of wild-type Rev and MTs **A.** Coomassie stained gel showing tubulin mass in the supernatant (S) and pellet (P) fraction when GMPCPP polymerized MT are incubated with varying concentration of wild-type Rev, in the presence of Mg^{++} . **B.** Graphical representation of the quantification of tubulin heterodimers released in the (S) fraction after incubation with varying concentration of wild-type Rev in the presence of Mg^{++} . **C.** Coomassie stained gel showing tubulin concentration in the (S) and (P) fraction when GMPCPP polymerized MT are incubated with varying concentration of wild-type Rev, in the absence of Mg^{++} . **D.** Graphical representation of the quantification of tubulin heterodimers released in the (S) fraction after incubation with varying concentration of wild-type Rev in the absence of Mg^{++} . Depolymerization is more efficient in the absence of Mg^{++} with $p=0.01$ (linear regression and Bonferroni corrections; $n=49$). Black stars represents $p=0.04$ (linear regression and Bonferroni corrections; $n=15$). Error bars correspond to standard deviations.

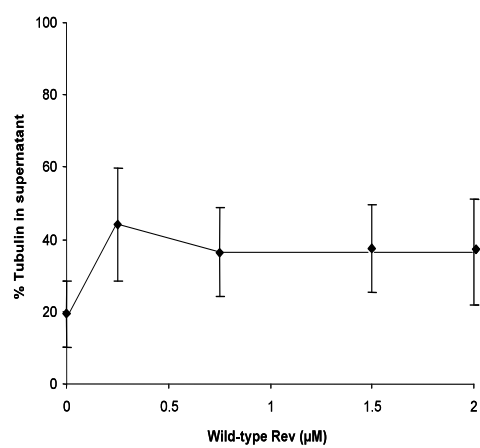
A



C



B



D

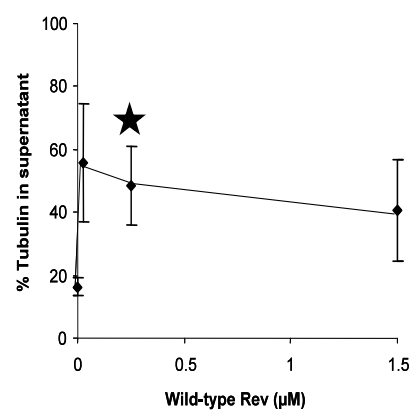
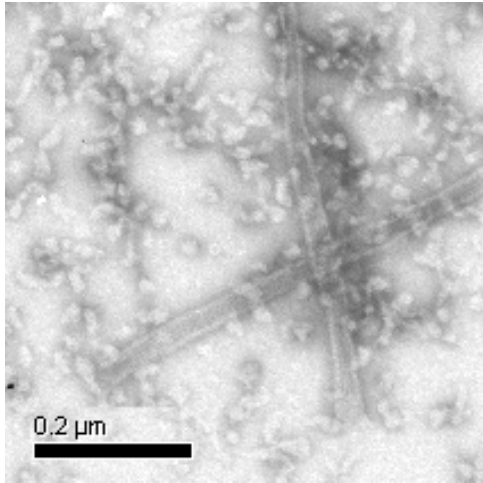
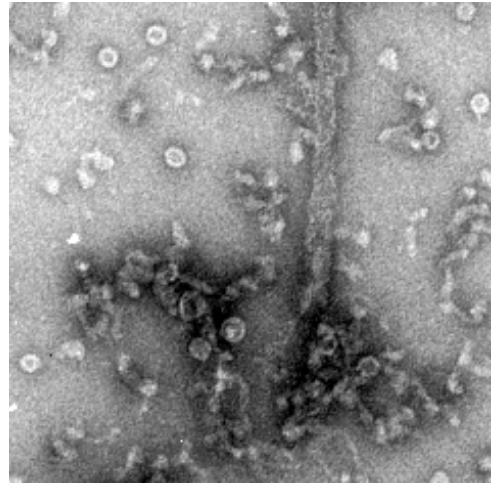


Figure 14: TEM analysis of Rev and its mutants with MTs. Electron micrographs of GMPCPP stabilized MTs treated with: **A.** Control buffer, **B.** Wild-type Rev, **C.** M4, **D.** M6. Black arrow in panel **D** points to the presence of M6 filament in the presence of MTs and are absent in case of wild-type Rev and M4. Magnification bar correspond to 200 nm.

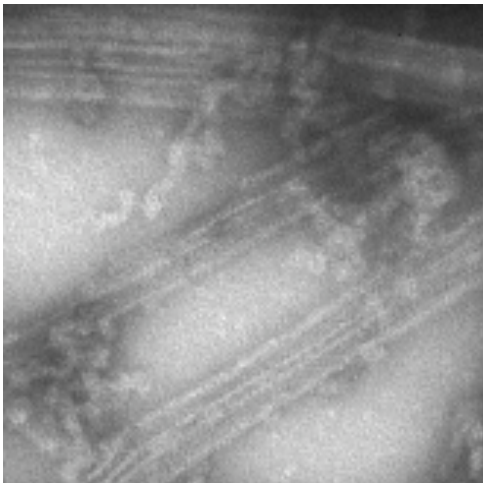
A.



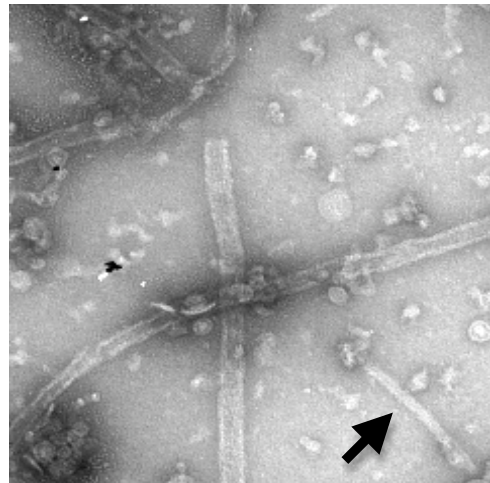
B



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D



-present in samples containing Rev compared to control samples (one-way ANOVA; $p < 0.001$) (Figure 15).

Measuring interactions between M4 and MTs

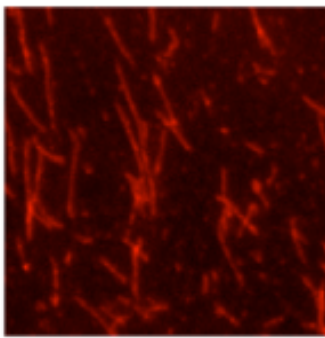
Sedimentation assays demonstrate that M4 is unable to depolymerize MTs both in the presence and absence of Mg^{++} (linear regression and Bonferroni correction; $p = 0.7$ and $p = 0.18$ respectively, Figure 16). Wild-type Rev depolymerized microtubules better than M4 (regression and Bonferroni correction; $p < 0.05$) (Figure 13) and fluorescence micrographs (Figure 15) show M4 causes MTs to bundle in contrast to wild-type Rev that depolymerizes MTs.

These data show that wild-type Rev and not M4 depolymerizes MTs. Conversely, M4 appears to stimulate MT bundling. This is consistent with the hypothesis that M4 mutation does not affect MT binding. Instead, the orientation of M4 binding is altered in a manner that allows MT cross-linking. These results are further consistent with the hypothesis that multimerization is important for MT depolymerization.

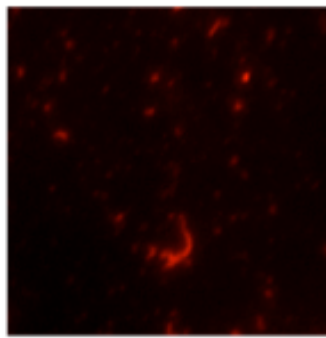
Measuring interaction between M6 and Tubulin heterodimers

The ability of M6 to form high molecular weight complexes with tubulin compared to wild-type Rev was evaluated using sedimentation assays. Because M6 does not purified as extensively as wild-type Rev, I wished to determine the ability of non-specific proteins present in bacterial cell extracts to interact with tubulin. Addition of this control extract (total protein quantity 11 ng, corresponding to equivalent amount of Rev in 3 μ M Rev) to tubulin heterodimers had no demonstrable interactions with tubulin as detected by the sedimentation assays (Figure 17). In the absence of Rev, tubulin heterodimers fractionate into the supernatant indicating that tubulin binding proteins are not present in control bacterial extracts. In contrast to

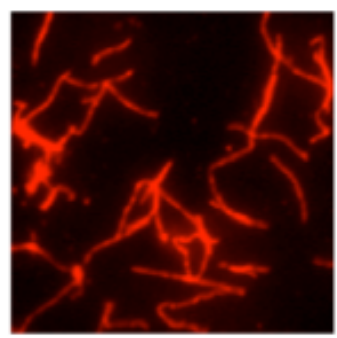
Figure 15: Immunofluorescence of wild-type Rev and M4 and MTs. **A.** Immunofluorescence micrographs showing **A.** MT of varying size in the absence of Rev stained with α -tubulin antibodies. **B.** MT reduced in length due to depolymerization in the presence of wild-type Rev. **C.** MT bundles in the presence of M4.



A



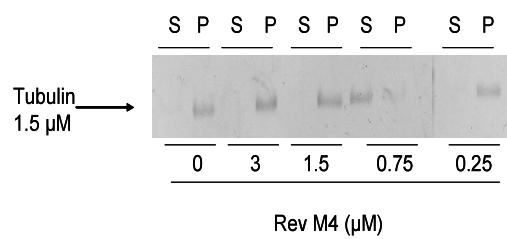
B



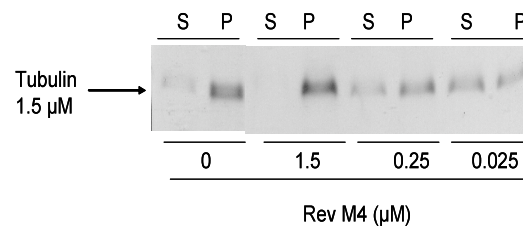
C

Figure 16: Sedimentation Assays of M4 with MTs. **A.** Coomassie stained gel showing tubulin concentration in the supernatant (S) and pellet (P) fractions when GMPCPP polymerized MT are incubated with varying concentrations of M4, in the presence of Mg^{++} . **B.** Graphical representation of the quantification of tubulin heterodimers released in the supernatant (S) fractions after incubation with varying concentrations of M4 in the presence of Mg^{++} . **C.** Coomassie stained gel showing tubulin concentration in the supernatant (S) and pellet (P) fraction when GMPCPP polymerized MT are incubated with varying concentration of M4, in the absence of Mg^{++} . **D.** Graphical representation of the quantification of tubulin heterodimers released in the supernatant (S) fractions after incubation with varying concentrations of M4 in the absence of Mg^{++} . Error bars correspond to standard deviations.

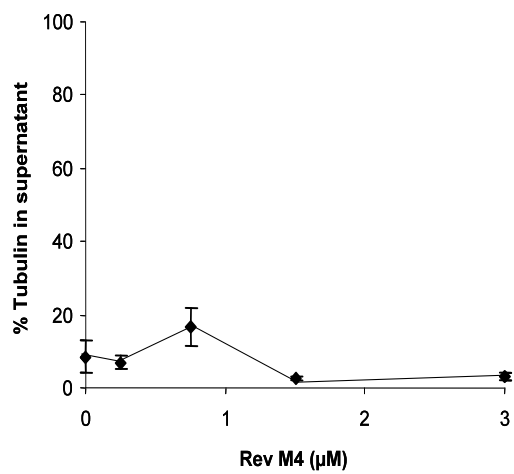
A



C



B



D

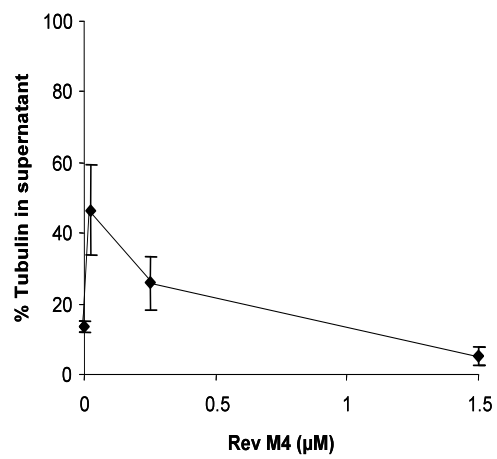
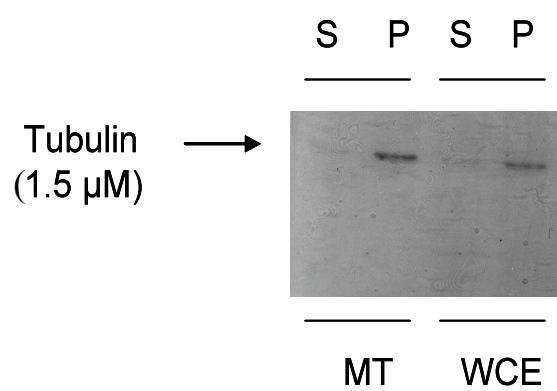
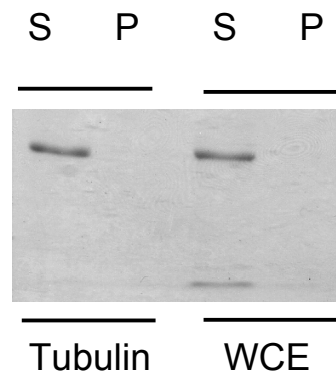


Figure 17: Effect of WCE on tubulin and MTs. A. Coomassie stained gel showing tubulin in supernatant (S) and pellet (P) fraction after high speed centrifugation (360,000 x g) of tubulin alone and tubulin treated with 11 ng of whole bacterial cell extract (WCE). **B.** Coomassie stained gel showing tubulin in supernatant (S) and pellet (P) fraction after high speed centrifugation (360,000 x g) of MT alone and MT treated with BL21(DE3) *E.coli* (WCE). **C.** Electron micrograph showing MT treated with WCE. **D.** Graphical representation of comparison showing the percentage of tubulin present in supernatant fraction after treatment with WCE, wild-type Rev and M6.

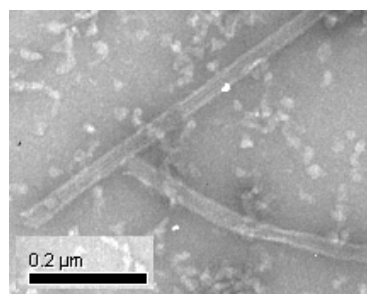
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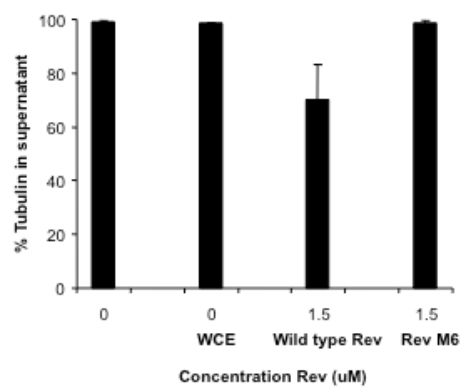
B



C



D



wild-type Rev, M6 did not lead tubulin to fractionate into pelleted fractions during sedimentation assay (student's t-test; $p=0.42$) (Figure 17, 18). TEM analysis of M6-tubulin mixtures however detected the presence of ringed structures similar to those formed by wild-type Rev (Figure 10). The sizes of these rings were smaller than those formed by wild-type Rev (Figure 19). Similar to wild-type Rev, analysis of 1:1 M6-tubulin heterodimer mixtures by S200 gel filtration shows that M6 forms high molecular weight complexes in the presence of Mg^{++} that elutes out in the void volume (Figure 8). In contrast to the results obtained with wild-type Rev, the stoichiometry of tubulin and M6 is altered and more heterogeneous than positive controls.

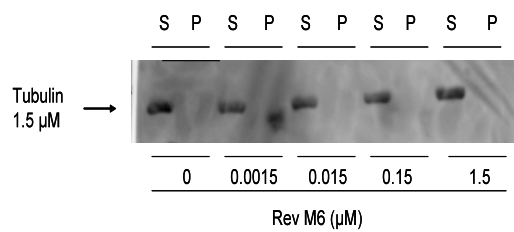
In the absence of Mg^{++} , sedimentation assays demonstrate that M6 did not form high molecular weight complexes in a significant manner (Student's t-test; $p=0.12$) (Figure 18). S200 chromatography however shows that M6 retains the ability to bind tubulin and forms heterogeneously sized complexes, ranging in size from 150 to 500 kDa (Figure 9). On the contrary, wild-type Rev forms complexes eluting in the fractions corresponding to ≈ 142 and 177 kDa.

Measuring interactions between M6 and MTs

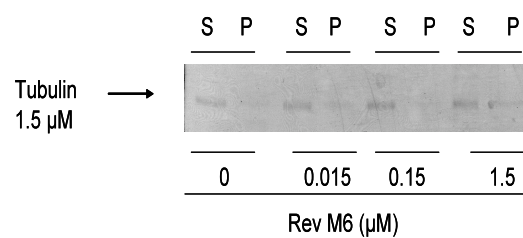
The ability of M6 to depolymerize MTs was analyzed by sedimentation assays where depolymerization was monitored by quantifying the amount of tubulin in the supernatant fraction. In contrast to wild-type Rev, M6 was unable to depolymerize MT both in the presence and absence of Mg^{++} (Student's t-test; $p=0.25$ and $p=0.34$ respectively) (Figure 20). MT in the presence of M6 appeared healthy as compared to those treated by wild-type Rev, as observed by TEM (Figure 14). MT peels and rings were rarely seen in presence of M6 showing very little

Figure 18: Sedimentation Assays of M6 with tubulin. **A.** Representative coomassie stained gel showing tubulin mass in the supernatant (S) and pellet fraction (P) when tubulin heterodimers are incubated with varying concentration of M6, in the presence of Mg^{++} . **B.** Graphical representation of the amount of tubulin present in supernatant (S) fractions after incubation with M6 (coomassie stained gel in **A**) in the presence of Mg^{++} . **C.** Coomassie stained gel showing tubulin concentration in the (S) and (P) fraction when tubulin heterodimers are incubated with varying concentration of M6, in the absence of Mg^{++} . **D.** Graphical representation of the amount of tubulin heterodimers present in supernatant (S) fractions after incubation with M6 in the absence of Mg^{++} . Error bars correspond to standard deviations.

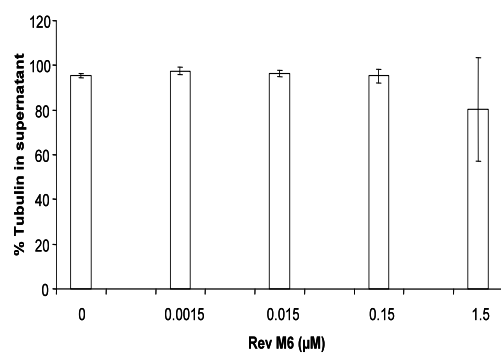
A



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B



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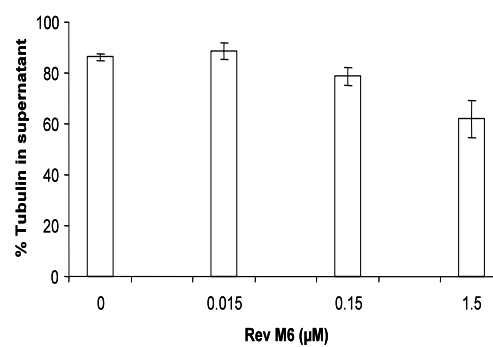


Figure 19: The diameters of RTTs formed by wild-type Rev and M6 are different. This graph plots the diameter of RTTs formed by wild-type Rev (n=81, black) or M6 (n=94, red) on the x-axis against the frequency of RTTs with a specific diameter (y-axis).

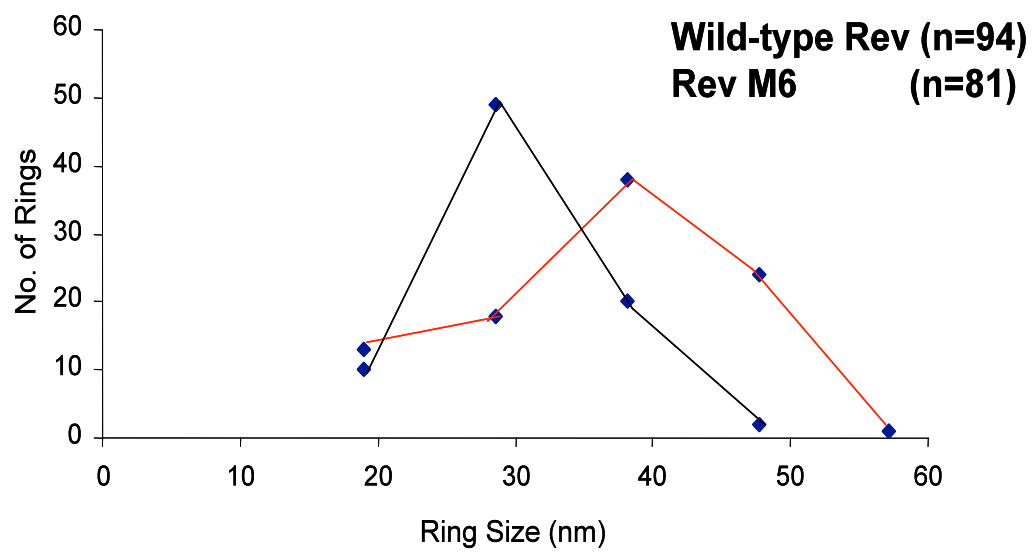
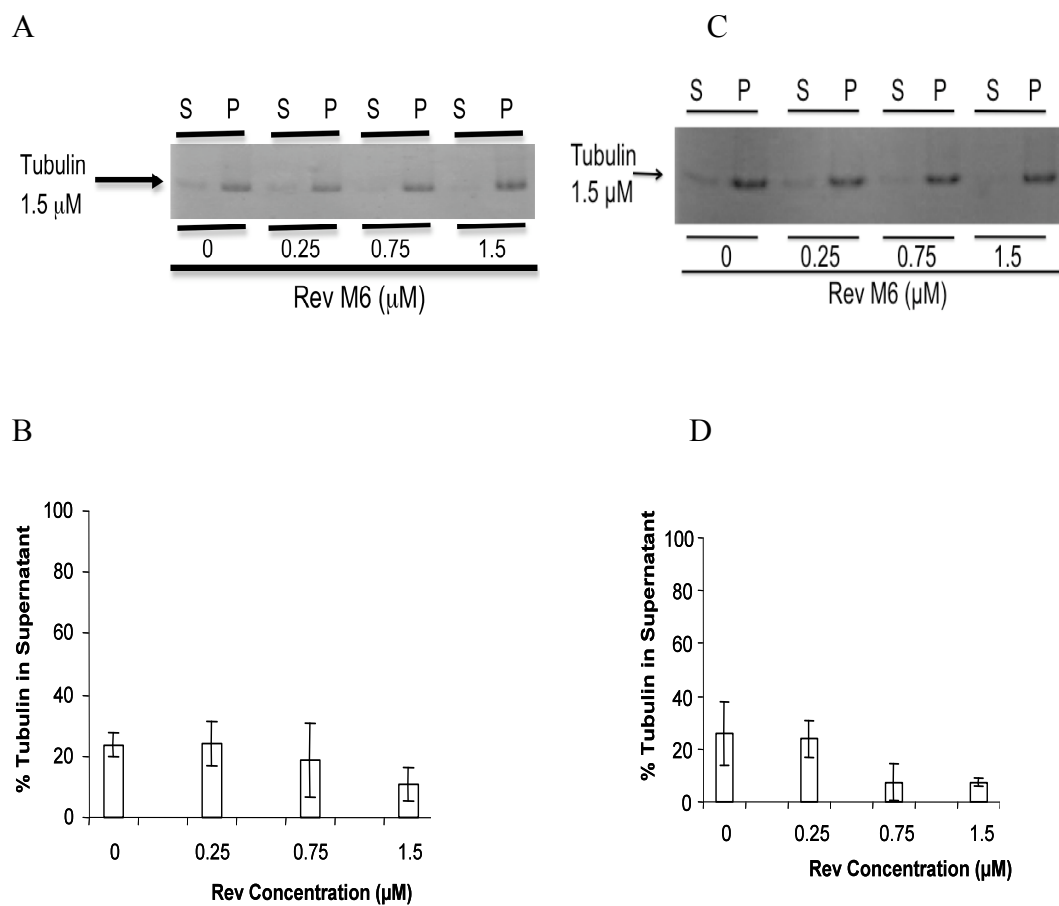


Figure 20: Sedimentation Assays of Rev M6 with MTs. **A.** Coomassie stained gel showing tubulin concentration in the supernatant (S) and pellet (P) fraction when GMPCPP polymerized MT are incubated with varying concentration of Rev M6, in the presence of Mg^{++} . **B.** Graphical representation of the quantification of tubulin heterodimers released in the (S) fraction after incubation with varying concentration of Rev (coomassie stained gel in **A**) in the presence of Mg^{++} . **C.** Coomassie stained gel showing tubulin concentration in the (S) and (P) fraction when GMPCPP polymerized MT are incubated with varying concentration of Rev M6, in the absence of Mg^{++} . **D.** Graphical representation of the quantification of tubulin heterodimers released in the (S) fraction after incubation with varying concentration of Rev (coomassie stained gel in **C**) in the absence of Mg^{++} .



sign of MT depolymerization. The occurrence of rings could be the results of M6 interacting with small amount of background tubulin being released from MT spontaneously, causing ring formation. Interestingly M6 filaments were also observed in the presence of MTs (Figure 14), suggesting deficiency in liberation of Rev from M6 filaments.

Taken together, the results demonstrate that deletion of four arginines reduces the ability of Rev to depolymerize MTs. The deletions also affect ring formation by M6, as rings formed by M6 are smaller in size compared to those formed by wild-type Rev.

DISCUSSION

Rev and filament formation

The long-term goal of this study was to study the mechanism underlying Rev-MT interactions. Table 2 summarizes the finding of this research. Specifically, one aim was to determine the role played by Rev-Rev interactions in RTT formation and MT depolymerization by comparing the ability of the M4 mutant to interact with tubulin relative to the wild-type protein. The second aim was to test the hypothesis that the ARM, specifically residues 41-44 altered by the M6 mutation are important for Rev-MT interactions, specifically involved in tubulin binding. These mutations were used because they have well-characterized biochemical and biological effects (Malim *et al.*, 1991; Battiste *et al.*, 1996; Brice *et al.*, 1999) and thus were a good place to start with Rev-MT studies. When its multimerization motifs are mutated, Rev lacks the ability to form high order multimers (Malim *et al.*, 1991; Daly *et al.*, 1993, 1995; Auer *et al.*, 1994; Thomas *et al.*, 1998; Brice *et al.*, 1999). In contrast, Rev's ARM is not involved in multimerization but is critical for RRE binding and nuclear import (Daelmans *et al.*, 2004).

This work began with protein purification and its polymerization into filaments. Although filament formation is known to be the best way to keep Rev functional in the absence of high salt (Watts *et al.*, 1998), it is not known whether Rev's ability to form filaments requires the presence of the ARM and the ability to multimerize. The results presented here show that wild-type Rev formed filaments similar in width (≈ 13 nm) to those described by Watts *et al.*, 1998. In the case of M4, no such filaments were observed, instead short oligomers were seen. The oligomers

Table 2: Summary of effect of Rev and its mutants on tubulin heterodimers and MTs.

Rev type	Sed. Assay		TEM		S200	Conclusion
	Tubulin Heterodimers	Microtubules	Tubulin Heterodimers	Microtubules		
Wild-type	Forms high mol. weight complex, does not require Mg ⁺⁺	Releases tubulin heterodimers, both in the presence and absence of Mg ⁺⁺	Forms RTTs	Releases tubulin heterodimers, Forms RTTs	Complex elutes in the void volume in the presence of Mg ⁺⁺	
M4	Form high mol. weight complex, requires Mg ⁺⁺	Deficient in depolymerization	Form high mol. weight complex, No RTTs	Deficient in depolymerization, Causes microtubule bundling	Complex elutes in the void volume in the presence of Mg ⁺⁺	Residues 23, 25, 26 required for RTT formation and microtubule depolymerization
M6	Does not form high mol. weight complex	Deficient in depolymerization	Forms smaller rings	Deficient in depolymerization,	Complex elutes in the void volume	Residues 41-44 important for RTT formation and microtubule depolymerization

were not straight as filaments formed by wild-type Rev suggesting that there is a conformational change in the way one M4 interacts with the other. The absence of filaments showed that mutating residues 23, 25 and 26 in M4 affects Rev's ability to multimerize that prevented filament formation. Although it is formally possible that M4 is misfolded, other labs have successfully purified functional protein using comparable methods (e.g., Brice *et al.*, 1999). The result that M4 forms complexes with tubulin heterodimers with comparable apparent molecular masses to those formed by wild-type Rev and tubulin suggests that the mutant protein retains the capacity to bind tubulin. This is consistent with the observation that M4 has negligible effects on RRE binding (Brice *et al.*, 1999). These results suggest that the inability of M4 to multimerize is due to mutation in Rev and does not depend upon the substrate with which it interacts.

Despite the fact that M6 could not be purified to the same extent as M4 or the wild-type protein, M6 was successfully refolded into stereotypical filaments despite the presence of impurities (Figure 5). These results suggest that arginines 41-44 deleted in M6 have a very limited role in filament formation.

TEM analysis shows that purified Rev is in form of distinct filaments and not aggregated. Results of gel filtration experiments described earlier that show Rev eluting out with tubulin heterodimers and TEM results showing Rev forming high molecular weight complexes with tubulin heterodimers also suggests that Rev used during the experiments is not aggregated.

Interaction between M4 and tubulin/MTs

Watts *et al.* (2000) previously showed that wild-type Rev protein could bind and depolymerize microtubules forming RTTs in the process. Their observation that Rev also forms RTTs when mixed with colchicine-treated heterodimers suggests that

the ability to form tubulin rings must lie with Rev since colchicine prevents tubulin polymerization. Therefore, if Rev is to join two different tubulin heterodimers together into a ring, then Rev must possess two binding sites. One will mediate the binding of one Rev monomer to a tubulin monomer present in the first of two adjacent heterodimers. The second site must then link to the adjacent tubulin heterodimer by binding either to a tubulin monomer or to a second Rev monomer bound to a tubulin monomer. The two models are distinguished by requirement of Rev multimerization, which at the very least must include dimerization.

Use of the M4 mutant affords an opportunity to distinguish between the two models. It has a well-documented effect on multimerization on the RRE while having only a small effect on RRE binding (Brice *et al.*, 1999). Moreover, the mutation is predicted not to interfere with Rev's ability to bind tubulin because the affected amino acids (#23, #25 and #26) lie outside the putative tubulin-binding domain. Although this binding site is not known with certainty, Watts *et al.* (2000) demonstrated that this activity likely resides in the *N*-terminal 59 amino acids and speculated that amino acids 34-59 are critical owing to a limited amino acid sequence similarity shared with Kinesin-13 proteins (Watts *et al.*, 2000). Data from both sedimentation assays (Figure 11), and size exclusion chromatography (Figure 8) showing that M4 readily forms high molecular weight complexes with tubulin ($M_r \geq 500$ kDa) are consistent with the hypothesis that M4 mutation does not perturb tubulin binding. This is also confirmed by fluorescence immunolocalization (Figure 15). If Rev binding is sufficient to depolymerize microtubules, then the M4 mutation should not affect microtubule depolymerization. The observations that the wild-type and not the mutant protein depolymerizes stabilized microtubules refute this hypothesis (Figures 13 and 16). Thus, it seems likely that Rev-Rev interactions are as important as Rev-tubulin

binding events. This is not surprising. It seems unlikely Rev has two separate binding sites for tubulin given its small size (116 amino acids) and that it is surprising that it has even one binding site given Rev's function during HIV infection.

Only a Rev dimer is logically required to link tubulin separate heterodimers into a RTT. There are several reasons for believing that Rev is acting within an oligomer larger than a dimer. First, the M4 mutation is not defective in dimerization, at least on its native target, the RRE (Brice *et al.*, 1999). Instead, the mutation appears to affect Rev's ability to form high molecular weight complexes. Second, Rev has a propensity to form long polymers in the presence and absence of the RRE (Heaphy *et al.*, 1991; Wingfield *et al.*, 1991; Watts *et al.*, 1998). Moreover, the estimated dimensions of a Rev monomer suggest it is sufficiently big to span the surface of a tubulin monomer within an RTT and maintain contact with adjacent Rev subunits (Wingfield *et al.*, 1991; Watts *et al.*, 1998; Watts *et al.*, 2000). Finally, no depolymerization was seen at stoichiometries as high as two Rev monomers per tubulin monomer (Figure 13). It seems likely that a significant number of microtubule ends would be bound by Rev and some depolymerization detected at this concentration. This conclusion may be tempered by the possibility that the M4 mutation greatly reduces its affinity for microtubule ends and would thus require higher M4 concentrations. This seems unlikely given that M4 interacts with tubulin heterodimers as well as the wild-type protein.

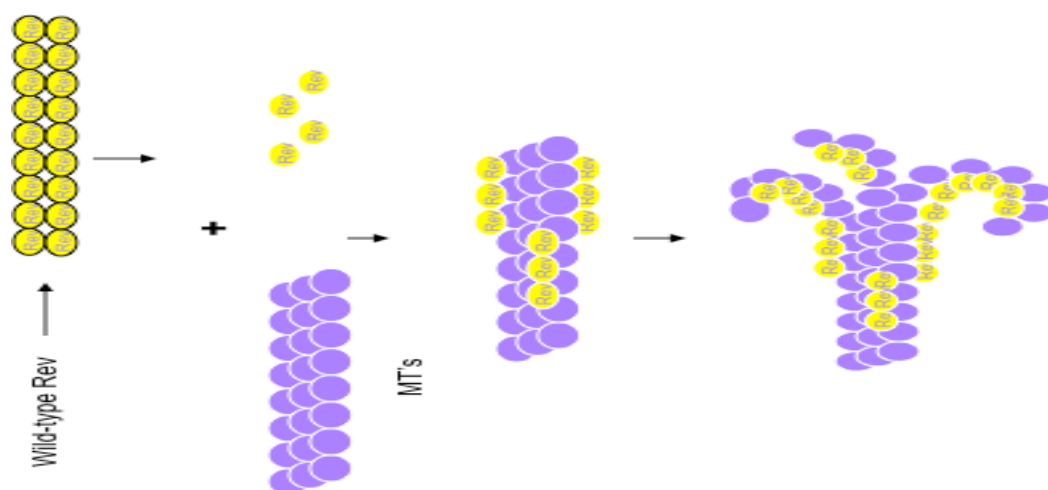
Rev multimerization may facilitate microtubule depolymerization by several mechanisms. Since Rev depolymerizes microtubules from their ends (Watts *et al.*, 2000), multimerization along the lattice can increase the rate tubule ends are bound. In contrast to Kinesin-13s that diffuse across the microtubule lattice (Ogawa *et al.*, 2004) and kinesin-8s that actively translocate toward the microtubule plus end before

stimulating depolymerization (Gupta *et al.*, 2006, Varga *et al.*, 2006), multimerization is a means Rev may target microtubule ends. How relevant this is will depend upon the extent multimerization is cooperative on the lattice. Indeed, Rev multimerization on the RRE is partially cooperative (Daly *et al.*, 1993; Zimmell *et al.*, 1996; Van Ryk and Venkatesan, 1999) and it can form polymers 40-120 nm long at moderate concentrations (40µg/ml) (Wingfield *et al.*, 1991).

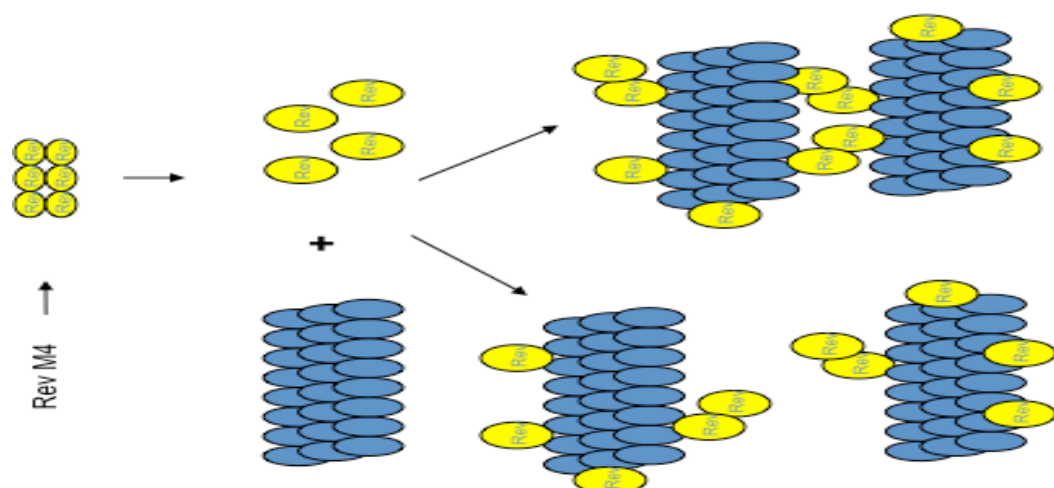
Multimerization may also be important for stabilizing curved tubulin conformations and/or exerting sufficient force to bend protofilaments. This seems likely given M4's inability to form RTTs with colchicine-treated heterodimers. Multimerization may be important in one of two ways (Figure 21). Multimerization across the microtubule surface would provide multiple contact sites in which the forces required to bend a protofilament might be deflected. This is precisely the mechanism employed by the kinesin-13s that make multiple contacts along the surface of the protofilament (Moore *et al.*, 2002; Hertzner *et al.*, 2003; Ogawa *et al.*, 2004, Shipley *et al.*, 2004). In this model, if M4 is altering the conformation of monomers within a Rev oligomer bound to a MT end, then the number of tubulin sites productively bound by Rev will decrease. The failure of M4 to depolymerize MTs is due to the failure to make the Rev oligomer sufficiently rigid to override the conformation of the intact MT.

Figure 21: Interaction between MTs and wild-type Rev and M4. **A.** Wild-type Rev binds and multimerizes on MT lattice and as it finds free MT ends, Rev oligomers stabilize the curved conformation of MT ends thus initiating RTTs formation. **B.** **(i)** M4 binds the MT lattice, **and (ii)** due to conformation change allows Rev monomers within an oligomer to bind tubulin out of the plane of the original bound MT (crosslinking), **or (iii)** alternatively there is no conformation change in M4 that still just forms oligomers, binds the MT lattice like a ‘caterpillar’ and causes adjacent MT to crosslink with binding site opposite to each other on M4.

A.



B.



This is supported by the observation that M4 and not the wild-type protein has a tendency to bundle microtubules. The ability to cross-link microtubules suggests that the M4 mutation is altering the orientation of Rev monomers with an oligomer such that MTs lying in a different plane can be bound. This would also explain why M4 is deficient in multimerization on the RRE. This capacity to generate mechanical force is reduced in case of M4 because of the lack of ability to form higher order multimers.

On the other hand it is also a possibility that the ability to multimerize may be arranging Rev spatially in such a way that MT binding sites are oriented in a manner to contact tubulin residues that control tubulin structure. Similar spatial arrangement is seen in case of the neck of MCAK that promotes disruption of lateral interaction between protofilaments and promotes MCAK diffusion on MT lattice. This ability being absent in M4 prevents it from depolymerization.

Interaction between M6 and tubulin/MTs

The ARM shares a sequence similarity with the catalytic domain of MCAK and is proposed to play an important role in MT binding and depolymerization (Watts *et al.*, 2000). If true, the M6 mutation should reduce tubulin binding, inhibit MT depolymerization, and block RTT formation. Indeed, high molecular weight complexes are not readily detected by sedimentation assays when M6 is mixed with colchicine-treated tubulin. Because these assays monitor complex formation, they do not differentiate between the possibilities that M6 affects tubulin binding or the formation of complexes after tubulin binding. Indeed RTTs are detected by TEM (Figure 10) albeit ring size is altered (Figure 19) suggesting that the M6 mutation does not eliminate tubulin binding. Smaller ring diameters may be due to more compact and tighter arrangement of Rev within RTTs possibly increasing the tension

across the ring and promoting ring instability. The deletion of four positive charged arginines along with the introduction of one negatively charged aspartic acid and hydrophobic leucine might affect the conformation of the tubulin binding site. S200 chromatography confirms M6 forms complexes with tubulin and that these complexes are different from complexes seen with the wild-type protein. M6 complexes in the presence and absence of Mg^{++} are smaller and heterogeneously sized (Figures 8 and 9). Taken together, these assays collectively suggest that this mutation reduces but not eliminates tubulin binding and alters complex formation and complex stability.

Surprisingly, the most compelling evidence that the M6 mutation affects tubulin binding comes from experiments where M6 is mixed with stabilized MTs. In experiments where Rev filaments are mixed with MT, substantial amounts of Rev filaments persist. Superficially, this could mean that M6 filaments are more stable than wild-type filaments and that Rev is unable to dissociate from the filament to interact with MT. However, because M6 filaments are never seen in the presence of tubulin heterodimers, this possible explanation is untenable. Thus, it appears that M6 has a reduced affinity for MTs than free tubulin. Because the exposed surface area of tubulin is reduced when it is in the MT polymer, it seems likely that M6 is capable of only binding MT ends.

Role of Magnesium in RTT formation

Watts *et al.* (2000) has shown that Rev forms high molecular complexes with Rev requires Mg^{++} . Experiments performed here are consistent with these findings. The role of Mg^{++} in RTT formation is unclear. Clearly, Mg^{++} by tubulin for binding guanine nucleotide. However, if MTs are first polymerized in the presence of Mg^{++} and then Rev is added but Mg^{++} concentrations are limiting, then RTTs do not form suggesting that Mg^{++} is also required for RTT formation. Although there is little

additional data to clarify the role of Mg^{++} , Figure 9 (the S200 chromatography-no Mg^{++}) suggests that the ability to form Rev-tubulin complexes in the absence of Mg^{++} is relieved by the M6 mutation. Thus, if the M6 mutant is capable of only binding MT ends, it seems plausible to suggest that Mg^{++} is important for binding MT lattices.

FUTURE EXPERIMENTS

Watts *et al.* (2000) have suggested a limited sequence similarity between Rev's ARM and the catalytic domain of MCAK (Figure 2) that extends above and beyond residues 41-44 mutated in M6. The role of these additional residues in Rev-MT interactions needs investigation. Earlier experiments characterizing Rev's multimerization has also shown involvement of additional residues in Rev multimerization-1. Analysis of the role of these residues in Rev's ability to form RTTs and MT depolymerization will further shed mechanistic insights in Rev-MT interaction.

SIGNIFICANCE

Studies conducted by Miyazaki *et al.* (1995) have shown that expression of Rev causes Cos7 cells to accumulate in G2/M phase of cell cycle. These effects were confirmed in HeLa cells and the cell cycle defect was localized to a point prior to the spindle assembly (N. Smith, personal communication). Moreover, these defects were also observed following expression of M4 and M6 although the severity of the defects was reduced (N. Smith, personal communication). Tubulin and Rev or Rev mutants can be detected in immunoprecipitates from whole cell extracts using tubulin- and Rev-specific antibodies indicating that both proteins physically interact in cells. Despite this, there is no compelling co-localization between Rev and MT suggesting that Rev must be interacting with tubulin heterodimers and not MTs (P. Kothalaxmi, personal communication). Since Rev accumulates perichromosomally during mitosis, it is spatially localized to inhibit MTs nucleating on the surface of chromatin through its interaction with heterodimers. This hypothesis is consistent with the results presented here showing that wild-type Rev, M4 and M6 each interact with heterodimers but only wild-type Rev is able to depolymerize stabilized MTs.

Rev's potential to be developed into an anti-MT agent is further reinforced as HIV infected cells are known to have large-scale MT pathologies. Although, MT pathologies observed during HIV infection has been attributed to viral protein-gp120 (Malorni *et al.*, 1997), the findings that Rev depolymerizes MTs and causes cell cycle defects suggests that gp120 may not be solely responsible and that Rev may also be responsible for these MT pathologies.

APPENDIX

Appendix A: Primer/oligonucleotide sequence

1) Rev mutant M4 (YSN_{23,25,26} → DDL)

Primer sequence:

F5' CGT CTG ATC AAA TTC CTG **GAC** CAG **GAT CTC** CCG CCA CCG AAC CCG 3'

R3' GCA GAC TAG TTT AAG GAC **CTG** GTC **CTA GAG** GGC GGT GGC TTG GGC 5'

2) Rev mutant M6 (RRRR₄₁₋₄₄ → DL)

Primer sequence :

F5' CAG GCG CGC CGT AAC **GAC CTT** TGG CGT GAA CGT CAG 3'

R3' GTC CGC GCG GCA TTG **CTG GAA** ACC GCA CTT GCA GTC 5'

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